ACCURATE AND SENSITIVE DETERMINATION OF SELECTED CONTAMINANTS FROM FOOD PACKAGING MATERIALS

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SUMMARY

This research project has investigated the migration of various types of toxic contaminants from food packaging materials into oily, aqueous and acidic food matrices. The first part of the project focuses largely on the development and optimization of various analytical methods for the investigation of bisphenolic analytes, namely bisphenol A (BPA), bisphenol A diglycidyl ether (BADGE), BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, bisphenol F (BPF), bisphenol F diglycidyl ether (BFDGE), BFDGE-$2H_2O$, BFDGE-2HCl in inner coatings of canned foods, as well as their migrational tendency into food using reversed phase high performance liquid chromatography (HPLC) with fluorescence detection. Acetonitrile was used to extract the analytes from the food matrix before subjecting the samples to liquid-liquid extraction, solid-phase extraction for further clean-up and preconcentration prior to HPLC analysis. The excellent validation data obtained suggests that this method can be applied to canned foods for the determination of migration of the eleven bisphenolic analytes from can coatings into food. Analytical results indicated that although migration levels of bisphenolics increased with storage time, the rates were different in different food matrices. Additionally, the type of food matrix influenced the major type of BADGE compounds present in the samples. The residual levels of the bisphenolic analytes present in the inner can coatings of thirty-five types of canned foods were also investigated; can tops, can bodies, and can bottoms were analyzed separately for their residual analyte content.

The extent of migration of all eleven analytes into the canned foods was examined in foods consisting of both solid and aqueous portions in a comparative analysis. The HPLC method was also transferred to the ultra-performance liquid chromatograph (UPLC ™) to allow for an improvement in separation efficiency, better chromatographic resolution and throughput. With the use of the UPLC, analytical run-time was improved by more than 300 %, and sensitivity of the various analytes was enhanced by more than 3 times.

During the liquid chromatographic analyses it was recognized that food matrices sometimes have interferences that hinder accurate chromatographic identification and quantitation. Therefore, a selective and specific method consisting of liquid chromatograph tandem mass spectrometry (LC-MS/MS) in multi-reaction monitoring mode was developed for the confirmation and quantitation of these bisphenolic analytes. The use of the LC-MS/MS methodology provided additional confidence and reliability for the identification of the analytes studies, with respect to the food interferences often present in food matrixes.

In the second part of the project, the migration of photoinitiators, such as benzophenone (BP), isopropyl-9H-thioxanthen-9-one (ITX), thioxanthen-9-one (TX), 2,4-dimethylthioxanthone (DMTX), and 2-chlorothioxanthen-9-one (CTX), from printed food packaging materials and beverages were also determined by the highly specific and sensitive LC-Tandem MS with electrospray ionization (ESI) using the multi-reaction monitoring mode. These photoinitiators are usually present in inks

applied to printed food packaging materials for functional purposes. Investigation of the ITX content in the food carton-boxes confirmed that ITX has been widely applied to the inks used in the food packaging material. The subsequent simultaneous analytical method developed for five photoinitiators, namely, benzophenone, isopropyl-9H-thioxanthen-9-one, thioxanthen-9-one, 2, 4-dimethylthioxanthone, and 2-chlorothioxanthen-9-one allowed for efficiency and convenience for food surveillance institutions.

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List of Abbreviations

1-HCPK	1-hydroxycyclohexyl-phenylketone
ASE	Accelerated solvent extraction
BADGE	Bisphenol A diglycidyl ether
BADGE-2H_2O	Bisphenol A bis(2,3-dihydroxypropyl)ether
BADGE-2HCl	Bisphenol A bis (3-chloro-2-hydroxypropyl) ether
BADGE-H_2O	Bisphenol A (2,3-dihydroxypropyl) glycidyl ether
BADGE-H_2O-HCl	Bisphenol A (3-chloro-2-hydroxypropyl)(2,3-dihydroxypropyl) ether
BADGE-HCl	Bisphenol A (3-chloro-2-hydroxypropyl)glycidyl ether
BFDGE	Bisphenol F diglycidyl ether
BFDGE-2H_2O	Bisphenol F bis(2,3-dihydroxypropyl)ether
BFDGE-2HCl	Bisphenol F bis (3-chloro-2-hydroxypropyl) ether
BfR	Bundesinsitit fuer Risikobewertung (Federal Institute for Risk Assessment)
BP	benzophenone
BPA	Bisphenol A [(2,2'bis(4-hydroxyphenyl)propane]
BPF	Bisphenol F
CE	Capillary electrophoresis
CE	Collision Energy
CTX	2-chlorothioxanthen-9-one
CV	Coefficient of variation
CXP	Collision Cell Exit Potential
D	Daltons
DMTX	2, 4-dimethylthioxanthone
DP	Declustering Potential
EC	European Commission
EDC	Endocrine disrupting chemicals
EFSA	European Food Safety Authority
EP	Entrance Potential
EPA	Environmental Protection Agency (United States of America)
ESI	Electrospray ionisation
EU	European Union
FAPAS	Food analysis proficiency assessment scheme
FCM	Food Contact Material
FP	Focusing Potential

FTIR	Fourier Transform Infrared (Spectrophotometry)
GC	Gas chromatography
GC-MS	Gas chromatography- mass spectrometry
HDPE	high density poly(ethylene)
HPLC	High performance liquid chromatography
HPLC-DAD/FLD	High performance liquid chromatography with diode array detection and fluorescence detection
HPTLC	High performance thin layer chromatography
ITX	isopropyl-9H-thioxanthen-9-one
ITX-d_7	2-isopropyl-[2H7]-thioxanthen-9-one
LC	Liquid chromatographs
LC-MS	Liquid chromatography mass spectrometry
LC-MS/MS	Liquid chromatography tandem mass spectrometry
LDPE	low density poly(ethylene)
LOD	Limit of Detection
LOQ	Limit of Quantitation
MDL	Method detection limit
MGEBPA	Monoglycidyl ether of BPA
MQL	Method quantitation limit
MRM	Multi-reaction monitoring mode
MS	Mass spectrometer
MS/MS	Tandem mass spectrometry
ND	Not Detected
NOGE	Novolac glycidyl ethers
PVC	Poly(vinyl chloride)
RP	Reversed Phase (HPLC)
rpm	Revolutions per minute
RSD	Relative standard deviation
S/N	Signal to noise ratio
SD	Standard deviation
SML	Specific migration limits
SPE	Solid phase extraction
TDI	Tolerable Daily Intake
TX	Thioxanthen-9-one
U(x)	Standard uncertainty
UPLC ™	Ultra Performance Liquid Chromatograph
UV	Ultra-violet (Radiation)
v/v	Volume-to-volume basis
VOC	Volatile organic compounds
w/w	Weight-to-weight basis

List of Publications

Journal Papers (Published)

1. C. Sun; L. P. Leong; P. J. Barlow; S. H. Chan, B. C. Bloodworth (2006). "Single laboratory validation of a method for the determination of Bisphenol A, Bisphenol A diglycidyl ether and its derivatives in canned foods by reversed-phase liquid chromatography". Journal of Chromatography A, 1129. 145–148.

2. C. Sun; S. H. Chan, B. C. Bloodworth (2007). "Determination of Isopropyl-9H-thioxanthen-9-one in Packaged Beverages by SPE Clean-up and Liquid Chromatography 5 with Tandem Mass Spectrometry Detection". Journal of Chromatography A, 1143. 145–148.

Journal Papers (Submitted or in progress)

1. C. Sun; Y. G. Chua; L. P. Leong; S. H. Chan, (2009). "Simultaneeous Determination of 5 Photoinitiators in Packaged Beverages by SPE Clean-up and Liquid Chromatography with Tandem Mass Spectrometry Detection". Journal of Chromatography A.

Conference papers

1. C. Sun; M. E. Grigg; J. S. H. Chan. (2004). "LC-MS/MS Analysis of Bisphenol-A Diglycidyl Ether (BADGE) and their Reaction Products in Canned Foods". **21st LC/MS Montreux Symposium**. Montreux, Switzerland. (Poster)

2. C. Sun ; P. J. Barlow ; S. H. Chan. (2005) "Migration of Toxic Contaminants from Canned Lacquers". **3rd NUS-HSA Annual Scientific Seminar**. Singapore. (Oral)

3. C. Sun; L. P. Leong; S. L. Poon-Yeo; S. H. Chan; B. C. Bloodworth. (2006) "HPLC analysis of bisphenol A diglycidyl ether, bisphenol F diglycidyl ether and their reaction products in canned coatings". **International Symposium on Chromatography.** Copenhagen, Denmark. (Poster)

4. C. Sun; S. H. Chan. (2006) "Determination of 2-Isopropyl thioxanthone (ITX) in food by SPE cleanup and liquid chromatography with tandem mass spectrometry detection" **120th AOAC Annual Meeting & Exposition**. Minneapolis, Minnesota, USA. (Poster)

5. C. Sun; L. P. Leong; S. H. Chan; B. C. Bloodworth. (2007) "Trace-Level Determination of 2-isopropyl-thioxanthen-9-one (ITX) in Food using ESI-LC-MS/MS"**. 4th NUS-HSA Annual Workshop**. Singapore. (Oral)

6. C. Sun; L. P. Leong; S. H. Chan; B. C. Bloodworth. (2007) "Migration of Toxic Contaminants from Canned Lacquers". **10th Asean Food Conference**. Subang Jaya, Kuala Lumpur, Malaysia. (Oral)

7. C. Sun; L. P. Leong; S. H. Chan; B. C. Bloodworth. (2007) "Simultaneous Method for the Determination of Bisphenol A, Bisphenol F, Bisphenol A Diglycidyl Ether, and Bisphenol F Diglycidyl Ether and their Derivatives in Canned Foods by ESI-LC-MS/MS". **Singapore International Chemistry Conference 5**. Singapore. (Oral & Poster)

8. C. Sun; L. P. Leong; S. H. Chan; B. C. Bloodworth. (2008) "A Fast Method for the Simultaneous Determination of Bisphenol A, Bisphenol F, Bisphenol A Diglycidyl Ether, and Bisphenol F Diglycidyl Ether and their derivatives in Canned Foods by Ultra-Performance Liquid Chromatography (UPLC ™)". **4th International Symposium on Food Packaging.** Prague, Czech Republic (Poster)

9. C. Sun; Y. G. Chua, Lai Peng Leong; S. H. Chan (2009) "Determination of Photoinitiators in Packaged Beverages by Solid Phase Extraction Clean-up and Liquid Chromatography with Tandem Mass Spectrometry Detection". **18th International Mass Spectrometry Conference**. Bremen, Germany.(Poster)

CHAPTER 1

Chapter 1
Introduction

1.1 Background

Food is packaged for a variety of reasons. It prevents food spoilage by protecting the contents against atmospheric conditions, micro-organisms, light, air, insects and rodents. Packaging also contributes to the improvement of nutrition and health. With proper packaging, loss of valuable nutrients will be kept to a minimal, and foods can also be transported without considerable damage from areas of excess to famine-stricken regions. More importantly, food packaging prevents losses of contents, and presents the food in an attractive form to the consumer [1].

A useful food packaging material is plastic. Plastic materials provide for the widest possible variety of crisp shapes and allows for greater detailing to be done during manufacture. They can often be manufactured quickly, using only a small amount of material, and offers cost benefits over glass and injection moulding [2]. However, the use of plastic in the production process generates more chemical wastes which often affects the environment.

Paper is another common material used in food packaging. The paper billboards the product, and makes aseptic paperboard packaging possible when laminated with plastic. These food packaging materials are also microwaveable, and may

contain a variety of geometric shapes. Unfortunately, they degrade quickly, and provide less barrier properties.

Metal food cans, first developed hundred and fifty years ago [3], is an excellent form of food packaging material as the material offers excellent barrier properties, and that sterilized food can be preserved for up to four years if sealed properly. Moreover, these food cans are well able to resist the wear and tear of storage and transportation. About 100 billion cans are produced annually worldwide for packing perishable food [4].

1.2 Coatings used in canning

The interior surfaces of food cans are usually coated with a layer of lacquer coating to improve its appearance and to prevent corrosion of the underlying metal can due to contact with moisture and dissolved air. This interior coating is very important as it also protects the bare metal from interactions with the food components. Flavor changes may result from the interaction of the coating components or from adsorption of flavor agents from the packed food into the coating. Therefore, as flavor can be affected by minute amounts of substances, high baking temperatures are usually used in order to drive out all residual solvents and other volatile flavor detractors. This means that the lacquer needs to be stable over a wide range of temperature and be able to resist the heat from the harsh canning processing conditions so that the durability of canned products can be enhanced. In addition, in food products rich in sulphur, such as fish, meat and certain vegetables, the lacquer serves to reduce unpleasant greyish

discolourations due to the formation of tin (II) sulphides from the reactions of the underlying tin and the sulphides in food [5].

Generally, the two most common can coatings applied are the epoxy phenolic resins, and the poly(vinylchloride) (PVC) organosols as they have highly crosslinked structures to withstand extreme processing conditions of 90 min at 121 ^{o}C.

A basic PVC organosol formulation usually incorporates a high molecular weight PVC organosol dispersion resin which is thermoplastic and extremely flexible. The adhesion of the coating may be improved by copolymerizing with polar reactants such as maleic acid and maleic anhydride. Plasticisers are also added to aid the film formation. As a result, highly flexible can coatings are formed, which are especially suitable for use in highly deformed components, cans with pull-off lids, and cans which are heavily shaped during the manufacturing process [3]. They also display good resistance to chemical attack, and are heat-sensitive [5].

1.2.1 Epoxy resins

Epoxy resins are oligomers containing at least two epoxy groups or two glycidyl groups which are able to participate in further crosslinking reactions [6]. Bisphenol A (BPA) is the most common hydroxyl-containing compound used in the synthesis of bisphenol A diglycidyl ethers (BADGE) to produce epoxy resins that have been used extensively in adhesives and protective coatings (Figure 1.1). In the context of food cans, they are mostly employed as epoxy-phenolics, whereby the hydroxyl

functionality in these resins carries the purpose of participating in crosslinking during curing reactions. The resulting coating displays the good adhesion properties of the epoxy together with high chemical and heat resistance.

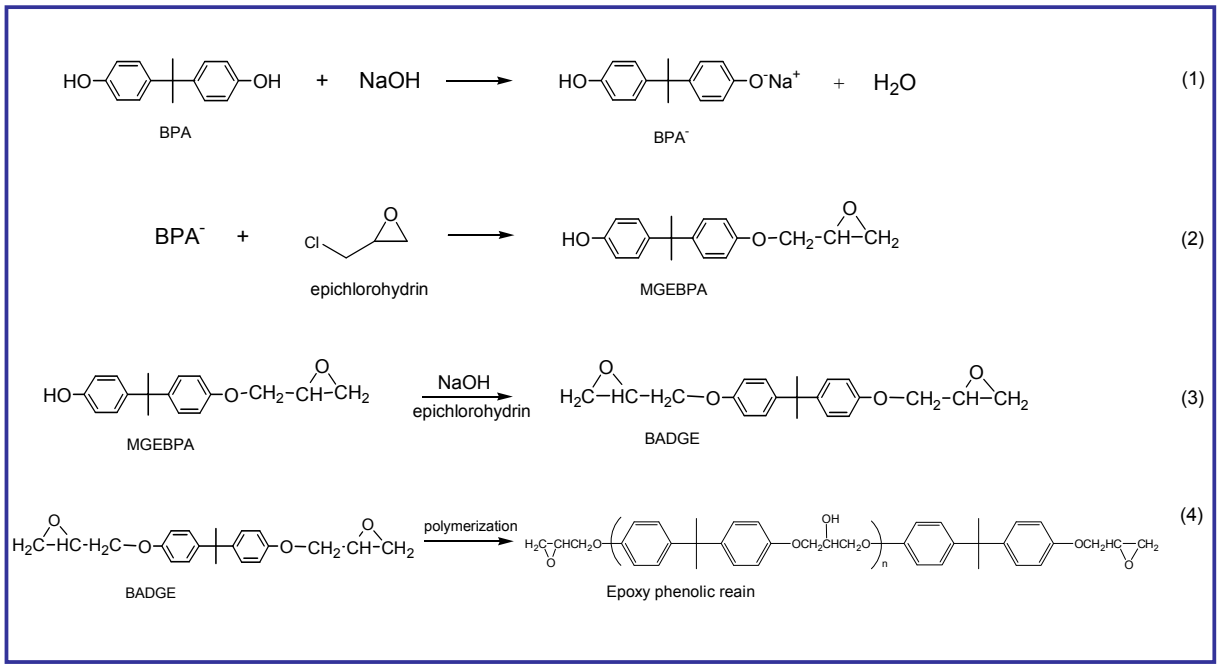

Figure 1.1 Formation of epoxy phenolic resins using BPA as starting material

As shown in Figure 1.1 above, under basic conditions, bisphenol A epoxy resins are synthesized by the reaction of bisphenol A and epichlorohydrin to form the BPA anion, BPA^-, which attacks epichlorohydrin and results in the formation of a new oxirane ring. This leads to the loss of the chloride anion, and results in the formation of the monoglycidyl ether of BPA (MGEBPA). Subsequent reactions of epichlorohydrin with the phenolic group of MGEBPA, in the presence of NaOH yields BADGE.

Similarly, bisphenol F (BPF) is used in the manufacture of bisphenol F diglycidyl ether (BFDGE) to produce epoxy novolac resins. Additionally, BADGE and BFDGE are also used as additives in the manufacture of poly(vinylchloride) (PVC) based organosols to scavenge for hydrogen chloride produced during the degradation of the organosols. As a result, residues of BPA, BPF, BADGE and BFDGE from incomplete polymerization processes of the epoxy-type resins and PVC organosols may potentially migrate into food, thus being a source of contamination. Once migration of BADGE and BFDGE into food has occurred, the epoxy functional groups of BADGE and BFDGE may react *in situ* with water and/or hydrochloric acid to produce hydrolysis and hydrochlorination products [7] (Figure 1.2).

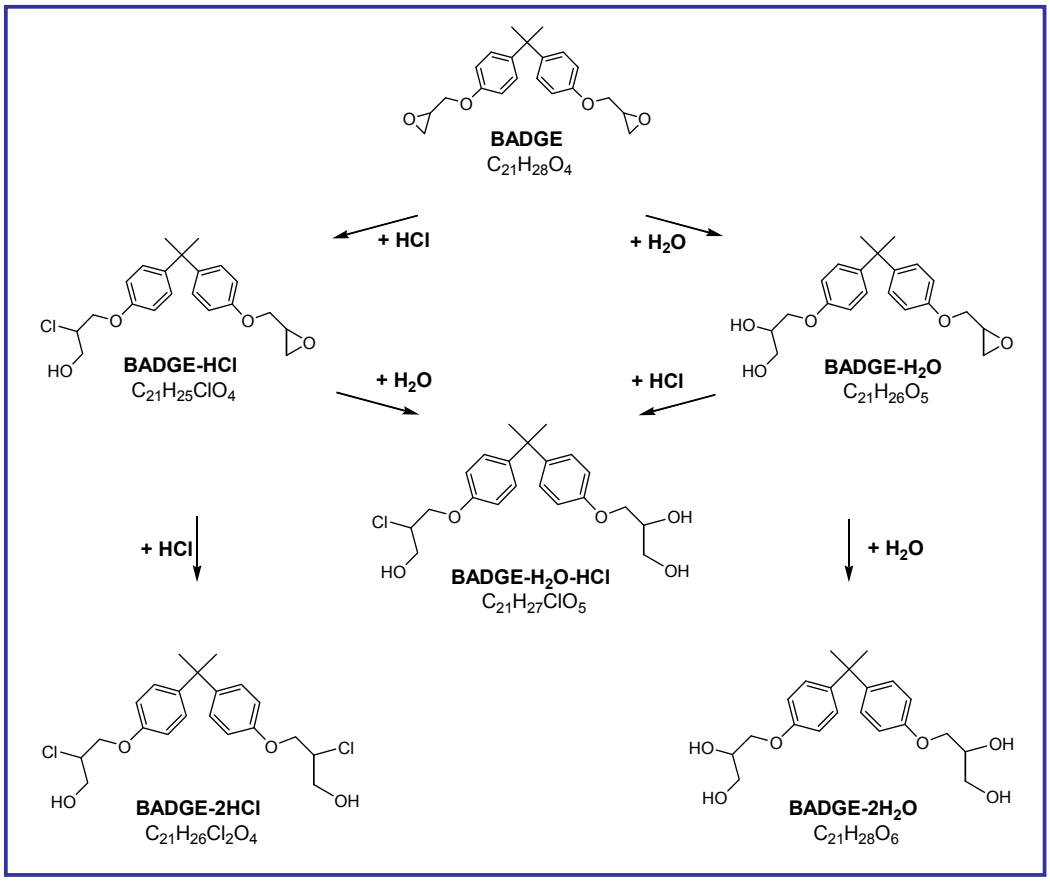

Figure 1.2 Reaction mechanisms of BADGE to form BADGE derivatives

1.2.2 Advantages of epoxy phenolic resins

BPA epoxy resins provide excellent adhesion to the can metal substrates. These resins have hydroxyl groups and ether groups along the chain, which can provide for interactions with the metal surface and other molecules in the coating. As the backbone of the epoxy resin consists of alternating flexible 1,3-glyceryl ether and rigid bisphenol A groups, it provides flexibility necessary for multiple adsorption of the hydroxyl groups on the surface of the metal, along with the rigidity to prevent adsorption of all of the hydroxyl groups. The remaining hydroxyl groups can therefore participate in cross-linking reactions, or hydrogen bond with the rest of the coating. These resins are especially resistant against aggressive can contents, and offer corrosion protection. However, even though they possess good chemical resistance, they have poor exterior durability and flexibility as compared to the PVC organosols.

1.2.3 Toxicology of bisphenolic compounds

Bisphenols belong to a group of endocrine disrupting chemicals (EDC) which are able to cause reproductive disorders due to their ability to mimic or antagonize the effect of endogeneous hormones, disrupt the synthesis and metabolism of endogenous hormones, or disrupt the synthesis and metabolism of hormone receptors [8]. Recent structure-activity studies showed that BPA, BPF and BADGE possessed endocrine modulating activity in MCF7 human breast cancer cells, promoted cell proliferation

and increased the synthesis and secretion of cell type-specific proteins [9]. Bisphenols, with two hydroxyl groups in the para position and an angular configuration are suitable for appropriate hydrogen bonding to the acceptor site of the estrogen receptor. When ranked by proliferative potency, it was observed that the longer the alkyl substituent at the bridging carbon of the bisphenols, the lower the concentration needed for maximal cell yield. This estrogenicity could be related to the ability of cellular enzymatic systems to break down these bonds and to generate molecules with free hydroxyl groups.

Studies have shown that BPA, produced in large quantities for the production of polycarbonate plastics and epoxy resins, can exhibit xenoestrogenic effects [10], cause the proliferation of breast cancer cells *in vitro* at very low doses of 6 μg/L [11], and affect other reproductive functions [12]. The potency of BPA generally ranged from 3 to 5 orders of magnitude lower than that of the natural hormone, estradiol [13]. Recently, valuable information regarding genetic differences in susceptibility to BPA [14], the effects on new BPA-target organs, as well as the undesirable effects on the prostate of the developing fetus [15] indicated that BPA appeared to be more estrogenic *in vivo* that earlier predicted in *in vitro* essays [16, 17].

BPA is liberated into the environment both accidentally and through permitted discharges [18]. Therefore, due to the widespread occurrence of bisphenol A in the environment, as well as its increasing industrial production in the recent years, potential exposure to these compounds are becoming a significant issue, and this has been a cause for concern for many regulatory agencies [19].

BADGE has been classified by the National Institute for Occupational Safety and Health as a tumorigen, mutagen and primary irritant [20]. Used as monomer of epoxy resins, BADGE was reported to become estrogenic at a high concentration (10 μM), even before hydrolytic treatment. Recently, epoxy compounds were reported as potential alkylating agents with possible specific cytotoxic actions in tissues affecting rates of cell division [21]. The toxicity depends mainly upon fractional concentration of the unreacted epoxy groups [22]. The hydrochlorinated-BADGE compounds are considered potentially toxic due to their structural resemblance to the genotoxic-chloropropanediols [23]. To further complicate matters, BADGE and BFDGE have short half-lives in acidic media that decreases further with increasing temperature, which suggests that the biological activity of the by-products of BADGE and BFDGE should also be considered when toxicity of the parent compounds are being assessed.

In 2004, the European Food Safety Authority (EFSA) further investigated into the safety of using BADGE in epoxy resins and vinylic organosols as can coatings in the light of recent toxicological studies. Mutagenicity studies performed using BADGE-2HCl indicated that gene mutations and structural chromosomal aberrations *in vitro* were not induced, although a weak positive response was observed in the *in vitro* micronucleus assay, in the absence of exogenous metabolic systems [24]. After considering supporting toxicological data, the specific migration limit has been adjusted to 9 mg/kg for the sum of BADGE, BADGE-H_2O, and BADGE-$2H_2O$, and 1 mg/kg for the sum of BADGE-HCl, BADGE-2HCl and BADGE-H_2O-HCl, respectively. Similarly, the specific migration limits for the BFDGE-analytes are set at 9 mg/kg for the sum of BFDGE, BADGE-H_2O, and BFDGE-$2H_2O$, and 1 mg/kg

for the sum of BFDGE-HCl, BADGE-2HCl and BFDGE-H_2O-HCl. The specific migration limits for BPA and BPF stands at 0.6 mg/kg of food each.

1.3 Determination of bisphenolic analytes from canned coatings in food

Due to the potential health effects of consuming the bisphenolic analytes, several research groups have developed various suitable analytical methodologies for the assessment of bisphenolic analytes in various types of canned foods as well as in food simulants. Generally, the reversed-phase high performance liquid chromatography (HPLC) technique using fluorescence detection was a common analytical tool for the determination of BPA [25-30]. In order to measure BPA and BPF simultaneously without the effects of interfering food components, the gas chromatography-mass spectrometry technique was also utilized, although prior chemical derivatisation of the analytes with acetic anhydride was necessary to improve the peak shapes and the robustness of the method [31].

Analysis of BADGE and their reaction products (BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, and BADGE-2HCl) have also been performed using HPLC [22, 26, 32, 33, 34]. Other analytical techniques available in the literature also included the use of normal-phase HPLC [34], and liquid chromatography-mass spectrometry [7, 10, 27, 28, 35]. However, even though these techniques were well suited for their intended analyses, there is currently no available analytical method suitable for simultaneously determining the wide range of bisphenolic analytes: BPA, BPF, BADGE and derivatives, as well as BFDGE and

derivatives in food matrixes. Table 1.1 lists some of the available results obtained by other research groups.

Table 1.1. Summary of results available from other research groups

Analyte(s) determined	Analytical method used	Limit of detection (µg/kg)	Reference
BPA	GC-MS	5	Goodson et al.[25]
	RP-HPLC	2	Munguia-Lopez et. al [226]
	RP-HPLC	10	Yoshida et al.[27]
	RP-HPLC	3	Kang et al.[28]
BPA & BPF	GC-MS	2 (BPA) 5 (BPF)	Goodson et. al [31]
BADGE, BFDGE	NP-HPLC	10 each	Biedermann et. al [34]
BADGE, BFDGE and all reaction products	RP-HPLC	30 (for BADGE and related compounds); 40 (for BFDGE and related compounds)	Food Standards Agency 2000 [22]
BADGE and reaction products	LC-MS	10 – 30 (for each BADGE-related product)	Petersen et. al [20]

1.4 Ink systems in food packaging

Apart from food cans as a useful food packaging material, paperboard packaging is also commonly used in the market to contain beverages, frozen food, cereals and other food products. These paperboard packaging are usually printed to improve visual appeal. A functional flexographic ink system used for food packaging purposes and other applications is required to display several properties. It should be able to achieve visual and colourful effects, adhere to the substrate, withstand external

conditions such as chemical exposure, abrasion, as well as extreme temperatures to which it is exposed to, and also achieve a consistent finished product. In order to fulfil these effects, a successful flexographic ink requires the composition of the following components: solvents, colorants, resins and additives [36].

1.4.1 Solvents

Solvents provide for fluidity, which is crucial for delivering the ink from the ink fountain to the substrate. They allow the ink to flow through the printing mechanism and evaporate to form a coating on the substrate. The solvent should adequately disperse or dissolve the solid components of the ink, while not reacting with the ink or any part of the press. In addition, it would be preferable for the solvent to dry quickly and thoroughly, emit low odour and possess minimal flammability and toxicity. Commonly used solvents include ethanol, methanol, propyl acetate and water [37]. For ultraviolet (UV) cured inks, fluidity is achieved by the liquid, uncured components of the inks, such as monomers.

1.4.2 Colorants

Colorants are compounds that absorb at certain wavelengths of visible light, and are classified into dyes or pigments in printing processes. Dyes are water-soluble and are usually basic, amino-based compounds. The strong colours, and transparent properties

of dyes make it valuable when transparency of the end product is desired. However, these dyes can be damaged by water and chemicals, and also have toxicity concerns.

Pigments are small insoluble particles, and have a wide range of properties since they can be made from a wide range of organic or inorganic compounds. In comparison with dyes, pigment containing inks are usually less prone to bleeding through the substrate, and are more chemical and heat resistant.

1.4.3 Resins

Resins are solid compounds that are soluble in the solvent and often have complex molecular structures. They allow the ink to adhere to the substrate, disperse the pigment and provide gloss to the finished coating. In addition, they can also impart differing degrees of flexibility, cohesive strength, block resistance and compatibility with the printing plates. Common categories of resins include polyamides, nitrocellulose, carboxylated acrylics, and polyketones.

1.4.4 Additives

Several components can be added to improve the performance of ink systems and the finished products. They include plasticizers, which improve the flexibility of resins; waxes, which enhance slip, rub and scuff resistance; wetting agents, which modify the

surface tension to improve adherence to the substrates; and defoaming agents, which reduce soap-like effects in water-based inks.

1.4.5 Types of ink systems

1.4.5.1 Solvent based inks

Solvent based inks were the first printing inks to be available commercially, and were widely used in many flexographic printing processes. They were considered the industry standard for ease of use and quality of printing as they dry quickly, and have high performance. However, as the solvents in solvent based inks are made up primarily of volatile organic compounds (VOCs) which are flammable, and contribute to the formation of ground-level ozone that causes health and respiratory problems, the resulting environmental concerns led to the development of other types of inks.

1.4.5.2 Water based inks

Although the primary solvent in water based ink is water, they can and often do contain varying percentages of organic solvents and VOCs. The colorants for water based inks are similar to those for solvent based inks, except that the resins and additives used are rather dissimilar. As water based inks are usually less flammable than solvent based inks, they are easier to store, and, depending on their VOC content,

they usually present less environment concerns. However, they take longer to dry and are not as easy to use as solvent based inks.

1.4.5.3 Ultraviolet (UV) cured inks

Ultraviolet (UV) cured inks are a relatively new technology in the flexographic printing industry. They are considerably different from the solvent and water based inks as they are cured through chemical reactions rather than drying through evaporation. In these chemical reactions, the presence(s) of three types of compounds are required: monomers, oligomers and photoinitiators. As a consequence, UV cured inks do not contain organic solvents, and this allows them to be free from VOC emission. However, they contain many chemicals which have not been fully tested for environmental and health concerns.

The photoinitiators in UV cured inks utilise UV radiation to initiate chemical reactions. In the presence of UV light, the photoinitiator fragments into reactive free radicals, which in turn react with the monomers and oligomers present to form a polymer that binds the ink together. Some of the more widely known photoinitiators used for food applications include benzophenone (BP), 1-hydroxycyclohexyl-phenylketone (1-HCPK), isopropyl-9H-thioxanthen-9-one (ITX), benzyldimethyl ketal, 2-ethylhexyl-4-dimethyl-amino benzoate, and 2-methyl-1(4-(methylthio)phenyl)2-morpholino propane-1-one [38].

1.5 Migration of contaminants from food contact materials (FCM)

Migration of chemicals can occur when food is placed in contact with a non-food material. Therefore, food packaging is an obvious example of a food contact material (FCM) as it can be in contact with food for a few minutes to several years.

Migration is defined as the mass transfer from an external source into food in physical contact with it by sub-microscopic processes, while negative migration is defined as the mass transfer from food into an external acceptor in physical contact with it by sub-microscopic processes [5]. Any substance in a food which is not normally a part of that food is considered a contaminant if its presence is unwanted. Hence, migrants into food can be regarded as contaminants, although in some regulatory systems they are called ‘accidental’ or ‘indirect’ additives or ‘adjuvants’ [5]. Migration species also includes the hydrolysis or decomposition of additives migrating into food caused by chemical sterilization and irradiation processes. Little is known about these reactions because they are very complex and occur only at trace levels.

In order to gain an insight about the migration profile of the migrants, it is necessary to first consider the fundamental mechanisms of mass transfer: diffusion and chemical reaction.

Diffusion is an important mechanism underlying migration. Any molecule above absolute zero temperature vibrates. At low energy levels, especially in structured solids, the movement is small and the individual molecule does not significantly

change position. However, when the molecules are in the liquid or gaseous state, they possess higher levels of energy and are capable of greater vibrational movement. Therefore, numerous collisions and direction changes occur in these two phases. The macroscopic movement of molecules is called 'diffusion' and is the underlying force behind migration.

Other than diffusion, chemical reactions can also be a route for an indirect mass transfer. The chemical reaction takes place at the interface between the food and the food contact material, after which the products of the chemical reaction dissolve in the food and are dispersed further by diffusion.

1.5.1 Migration of monomers / additives from polymers used in food contact materials

Polymers invariably contain some residual monomers from the incomplete polymerization processes during the manufacturing processes, and additives such as antioxidizing agents, plasticizers, lubricants and stabilizing agents. These additives have been added deliberately to change or to improve the physical, chemical, or mechanical properties of the polymer so as to optimize its manufacturing and use. These monomers and other products may also be formed from the polymers during thermal degradation, during packaging manufacture or during sterilization at high temperatures. As the molecular sizes of these monomers are small, the molecules are able to move through the polymer, even in the absence of external forces such as vibration. Therefore, the diffusion process can occur as long as the chemical potential

of the substances is different [39]. The diffusion process is also affected by the solubility of the monomer with the type of food medium it is in contact with, as well as the absorption capacity of the packaged product.

1.6 State-of-the-art analytical methods for determining amount of contaminants from food packaging materials

1.6.1 Ultra-performance Liquid Chromatography (UPLC^{TM})

In the recent years, manufacturers of liquid chromatography materials and equipment have introduced new stationary phases, new column geometries and new instruments in order to meet the increasing demand for the improvement of productivity and reduction of costs in analytical chemistry laboratories, while maintaining high resolution and analytical sensitivities.

The use of the sub-micron porous particles allows for a significant improvement to the resolution per unit time, as chromatographic efficiency (N) and optimal mobile phase velocity (u_{opt}) are both inversely proportional to the particle size (d_p). Therefore, due to the high efficiency of the sub-2 μm particles available commercially, column lengths can now be reduced in order to obtain similar resolution in a reduced analytical run-time. In addition, coupled with the increase in the mobile phase flow rate, the analytical run-time can be shortened even further, bringing about an increase in throughput for the laboratory. However, small particle sizes can cause a large high

pressure drop, as the former is inversely proportional to the square of the particle size: d_p^2:

(Equation 1.1)

$$\Delta P = \phi x \frac{\eta x L x u}{d_p^{\,2}}$$

Where η is the mobile phase viscosity, L is the column length, u is the mobile phase linear velocity, d_p is the particle size of the stationary phase, and Φ is the flow resistance [40]. This is the reason why the supporting liquid chromatographs are required to be able to withstand very high pressures from the stationary phases in order to be compatible with these stationary phases.

Since 2004, a new generation of stationary phases consisting of small porous particles with particle sizes of sub-2 μm that can withstand very high pressures (up to 1000 bar), and the corresponding liquid chromatographs have been commercialised from several suppliers under the trade name: ultra performance liquid chromatograph (UPLC). This was the first fast LC to be commercialised. To date, several other manufacturers have also produced similar types of fast LCs, under various other trade names that make use of similar technology. The UPLC instrument will be utilised in one of the sections of the research project for the determination of BADGE, BFDGE and their derivatives in Chapter 4.

1.6.2 Liquid Chromatography Tandem MS (LC-MS/MS)

In order to analyze a complex mixture more accurately with reference to their molecular weights, a separation technique, i.e., liquid chromatography (LC) or gas chromatography (GC), or capillary electrophoresis (CE) is coupled with the mass spectrometer (MS). Liquid chromatography (LC) is used for compounds that are not volatile, and not as suitable for gas chromatography (GC).

The most obvious advantage drawn from coupling a separation technique with mass spectrometry consists in obtaining a mass spectrum used for identifying the isolated product. Therefore, the ideal detector should preferably possess the following capabilities.

1. Have no alteration of the chromatographic resolution, which means not producing within the detector a mixture of products separated before the detection.
2. Have the highest possible sensitivity.
3. Be universal – capable of detecting all the eluted products.
4. Furnish the maximum structural information possible, and possibly allowing the positive identification of all the eluted components.
5. Be selective, i.e., to allow the identification of target products in a mixture.
6. Provide signals proportional to the concentration of the analytes.
7. Have a constant, or a predictable response factor.
8. Have a low cost/ performance ratio.
9. Not be harmful to the product.

10. Allow the deconvolution of chromatographic peaks, i.e., the decomposition of unresolved peaks into constituents.

However, the coupling of LC to the mass spectrometer is normally more delicate, as gas-phased ions need to be produced for mass spectrometer analysis, and that the elution solvent needs to be eliminated.

1.6.2.1 Electrospray Ionisation (ESI)

The ESI allows for very high sensitivity to be reached, and is easily coupled to the high performance liquid chromatography (HPLC), micro-HPLC and capillary electrophoresis.

An electrospray is produced by applying a strong electric field, under atmospheric pressure, to a liquid passing through a capillary tube with a weak flux (normally 1 – 10 μL/min). The electric field is obtained by applying a potential difference of 3 – 6 kV between this capillary and the counter-electrode, separated by 0.3 – 2 cm, producing an electric field that induces a charge accumulation at the liquid surface located at the end of the capillary, which will break to form highly charged droplets. A gas injected coaxially at a low flow rate allows the dispersion of the spray to be limited in space. These droplets then pass through either a curtain of heated inert gas, most often nitrogen, or through a heated capillary to remove the last solvent molecules. The solvent contained in the droplets evaporates, which causes them to shrink to the point where the repelling coulombic forces come close to their cohesion

forces, thereby causing their division. These droplets then undergo a cascade of ruptures, yielding smaller and smaller droplets. Each rupture yields two droplets of similar size, but under the effect of the strong electrical field, many smaller, highly charged droplets are produced too. When the electric field on their surface becomes large enough, desorption of ions from the surface occurs. A charge in excess accumulates at the surface of the droplet. Desorption of charged molecules whose concentration at the surface is higher. The formation of ions therefore is the result of the electrochemical process and of the accumulation of charges in the droplets. Small molecules less than 1000 D normally produce monocharged ions. The ESI can also be used in cases where molecules lack any ionisable site, through the formation of sodium, potassium, ammonium, chlorine, acetate or other adducts.

1.6.2.2 Tandem Mass Spectrometry

Tandem mass spectrometry, abbreviated MS/MS, refers to a general method that involves at least two stages of mass analysis, either in conjunction with a dissociation process, or a chemical reaction that causes a change in the mass or charge of an ion.

In the most common MS/MS experiment, a first analyzer is used to isolate a precursor ion (m_p^+), which then undergoes spontaneously or by some activation a fragmentation to yield product ions (m_f^+) and neutral fragments(m_n):

$$m_p^+ \longrightarrow m_f^+ + m_n$$

A second spectrometer analyses the product ions. This selective detection of the precursor ion/ product ion mass pairs (otherwise known as parent/daughter mass pairs) is useful in determining the analyte compounds with selectivity and specificity, as sample matrix is eliminated during the selection of the appropriate mass pair(s). In doing so, it is also possible for the analysis to reach very low detection levels of sub-ppb, since matrix interference is largely eliminated from the mass analysis.

1.7 Objectives of the research work

Due to the potential undesirable health effects resulting from the exposure to the bisphenolic analytes, as well as other undesirable food contaminants, it is critical to investigate the extent of the migration of the contaminants into food in an effort to safeguard public health. The results of the research will also allow regulatory bodies to assess the background levels of contaminants in packaged foods (canned foods and beverages contained in cartonboard packages) available in the local market for comparison with results obtained from other countries. Consequently, these migration results will function as a valuable resource for the implementation of food safety policies.

This thesis will cover two broad areas of contaminants from food contact materials:

1. Determination of the migration of bisphenolic monomers from canned coatings into food, using a variety of instrumental analytical methods.

2. Determination of the content of photoinitiators in beverages and their packaging material from printing inks applied onto paperboard packaging.

The proposed research is divided into eight sections. In the first section, development and optimization of a suitable analytical method capable for a preliminary investigation of the levels of chemical migration of BPA, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, and BADGE-2HCl from can coatings into various canned foods will be performed. This will allow us to better understand which types of bisphenolic contaminants are present in the food, and provide the rationale for further exploratory studies in this area.

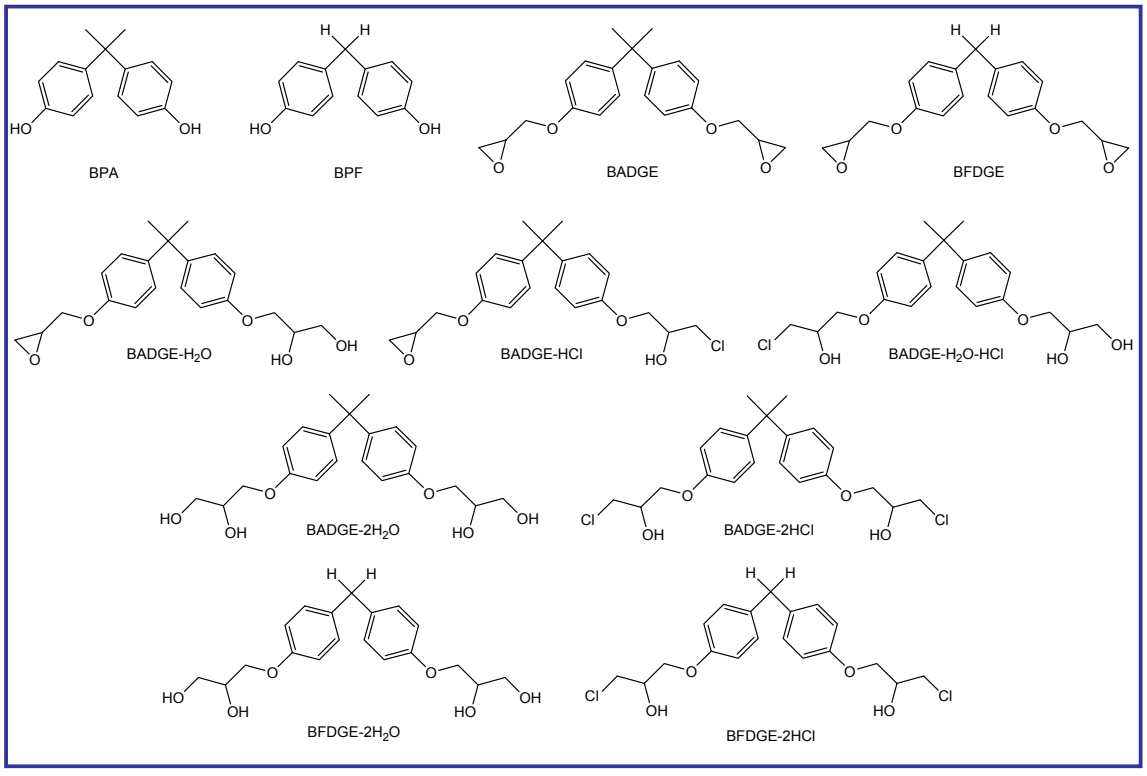

Figure 1.3 List of all bisphenolic analytes in this study

In the second section, an extensive migration survey of bisphenolic contaminants in various canned meat samples available in Singapore is conducted. In order to have a complete view of the bisphenolic contamination status in canned foods, the investigation not only targets the seven principle analytes, but includes another class of bisphenolic contaminants occasionally present in canned food, namely, bisphenol F (BPF), bisphenol F diglycidyl ether (BFDGE), BFDGE-2H_2O and BFDGE-2HCl (Figure 1.3).

The objective of the third section is to enhance the throughput of the method by improving upon the instrumental conditions of the liquid chromatograph. Although the HPLC technique was able to separate the 11 analytes satisfactorily, the instrumental run time of 95 min was far too long, and could be further improved upon using the recently introduced ultra-performance liquid chromatograph (UPLC ^{TM}). With the help of the UPLC system and the sub-2 μm hybrid particle sizes (1.7 μm) of their analytical columns, the throughput was improved by more than six times as the analytical run time was reduced to a mere 15 min. In addition to the shortened analytical run time, the sensitivity of the UPLC system was increased 3 times; solvent usage and operating costs were also significantly reduced with the improvement of the analytical method. The measurement uncertainty of the result was also determined with the available UPLC method validation results.

The need for confirmation of these structurally similar analytes in complex food matrices provides the basis of the fourth section of the research, where this thesis report the results from a study on method optimization of the bisphenol A- and

bisphenol F derivatives by positive and negative mode ESI- HPLC tandem MS, using multi-reaction monitoring (MRM) mode. The development of this selective and specific method was crucial for the confirmation and quantitation of all these bisphenolic analytes, where interferences may occur to hinder accurate chromatographic identification and quantitation.

The fifth section of the research focuses on the determination of the measurement uncertainty of the HPLC method that was developed for all the eleven analytes: BPA, BPF, BADGE and BFDGE, and their derivatives. This would ensure that the analytical data determined is technically sound and defensible, especially when there are cases of disputes between inter-laboratories, and also allow for the improvement of confidence associated with the reliability of analytical results of food samples, that can allow for the removal of barriers to international trade.

The sixth section of the research focuses on a totally different food contaminants area. Currently, inks applied to food packaging materials are not covered by specific European legislation, however, materials and articles intended to come into contact with foods should comply with the general criteria laid down in Article 3 of Regulation (EC) No. 1935/2004, stating that materials and articles in contact with food shall be manufactured in such a way that they do not transfer their constituents to food in quantities which could change the composition of the food or bring about unacceptable deterioration in the organoleptic characteristics thereof [41]. As a photo-initiator in UV cured inks, isopropyl-9H-thioxanthen-9-one (ITX), as well as other photointiators commonly used for food application, triggers the radical polymerization of the acrylic component of such inks to allow the liquid ink film to

dry. This investigation on photoinitiators migrating from printed packaged beverages was initiated in response to the recent food survey in France, Italy, Spain and Portugal in November 2005. In addition to the full method validation, the measurement uncertainty of the result would also be determined in the relevant chapters.

The last section of the research on toxic contaminants from food packaging materials focuses on the development of a simple, yet rapid analytical method capable for the specific detection and quantitation of a range of photoinitiators that may be used in formulations for UV-inks in food packaging applications, which consists of benzophenone (BP), 1-hydroxycyclohexyl-phenylketone (1-HCPK), isopropyl-9H-thioxanthen-9-one (ITX), thioxanthen-9-one (TX), 2,4-dimethylthioxanthone (DMTX), 2-chlorothioxanthen-9-one (CTX) (Figure 1.4). This analytical method has been validated on packaged milk and fruit juice beverages which are commonly packaged using heavily printed paper. Low method detection limits (15 – 20 μg/L for fruit juice and milk matrices), and method quantitation limits (50 μg/L for fruit juice; 35 – 50 μg/L for milk) were attained from this overall analytical method. The excellent validation data suggested that trace determination of the 5 photoinitiators could be performed in fruit juice and milk as well as their respective food packaging material.

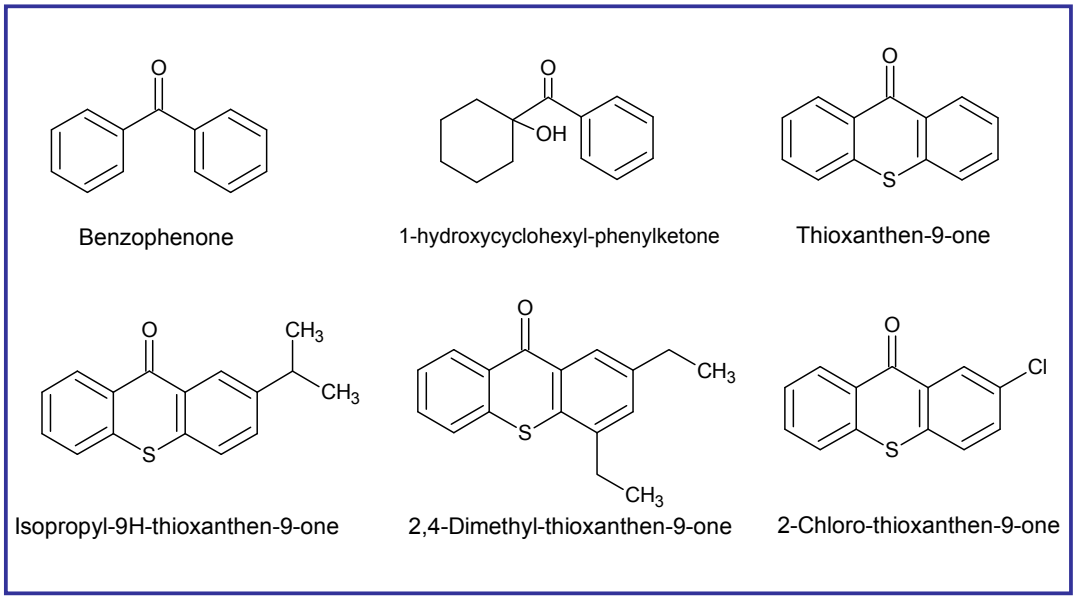

Figure 1.4 Chemical structures of the range of photoinitiators used in the study using LC-MS/MS

The objectives of the research reported here were:

- To develop, optimize and validate a suitable sample preparation method for selective analysis of the bisphenolic analytes in food.

- To investigate the variation of canned food samples to determine the homogeneity of chemical migration in canned foods processed on the same manufacture date. For this investigation, ten cans of each food medium type will be sampled to obtain statistically sound results.

- To study the effects of storage time and the type of food media (oily, aqueous and acidic nature) on the extent of migration activity.
- To determine the residual levels of BPA, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BPF, BFDGE, BFDGE-$2H_2O$, and BFDGE-2HCl present in the inner can coatings of thirty-

five types of canned foods; can tops, can bodies, and can bottoms will be analyzed separately for their residual analyte content.

- To compare the extent of migration of analytes in the food; solid portions and liquid portions of foods will be subjected to further comparative analysis where applicable.

- To improve upon the sensitivity and the throughput of the analytical method using UPLC ™, and to reduce the operating costs and solvent usage associated with the analysis.

- To develop a selective LC-MS/MS method for the confirmation and quantitation of all these bisphenolic analytes, especially in complicated food matrices; the specificity of the MRM-mass pairs during the analysis have important consequences for food regulatory agencies for the enforcement of the relevant food laws.

- To determine the measurement uncertainty associated with the HPLC analysis to obtain the standard uncertainty of the final analytical result(s).

- To develop optimize and validate a suitable sample preparation method for the sensitive and selective analysis of the photoinitiator, ITX in packaged beverages by LC tandem MS.

- To survey the ITX content in a range of packaged beverages and their respective printed food packaging materials.

- To determine the measurement uncertainty associated with the measurements of ITX in beveraged foods using the instrumental technique, LC tandem MS.

- To develop, optimize and validate a suitable sample preparation method for the simultaneous analyses of a novel range of photoinitiators (benzophenone, 1-hydroxycyclohexyl-phenylketone (1-HCPK), isopropyl-9H-thioxanthen-9-one (ITX), thioxanthen-9-one (TX), 2, 4-dimethylthioxanthone (DMTX), 2-chlorothioxanthen-9-one (CTX)) in packaged beverages by LC tandem MS. The specificity of the MRM-mass pairs during the analysis are particularly useful for food regulatory agencies in the enforcement of the relevant food laws.

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CHAPTER 2

Chapter 2

Optimisation of Bisphenol A, Bisphenol A Diglycidyl Ether and its derivatives in Canned Food by HPLC

2.1 Introduction

Bisphenol A (2,2'bis(4-hydroxyphenyl)propane, BPA) is used in the manufacture of bisphenol A diglycidyl ether (BADGE) which is then used for can coatings. The European Union has recently adjusted the specific migration limit of these compounds in food due to migration from can coatings [1,2], and as a result, many analytical methods have been developed for such migration studies [3-6]. The method reported in this chapter is an improvement of the methodology reported by Leepipatpiboon *et al* [6] involving an additional analyte, BPA. It illustrates the optimization of the sample extraction protocol step-by-step, along with its corresponding results. The reported method therefore consists of the optimum conditions of each sample extraction optimization step described.

In addition to the method optimization details, this chapter also reports the method validation studies of linearity, precision, accuracy and robustness. It was found that this method not only provided a robust analytical method capable of determining seven bisphenolic analytes simultaneously, namely, BPA, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, and BADGE-2HCl [Figure 2.1]

from can coatings into food, it also gave better recoveries of the various analytes than the published paper by Leepipatpiboon *et al* [6].

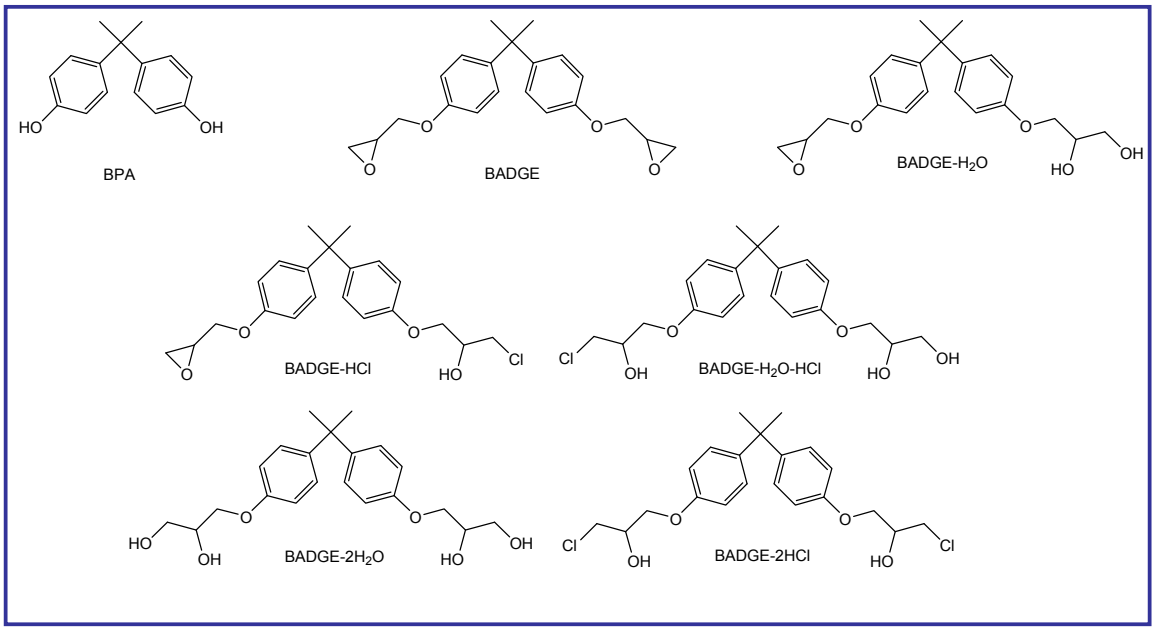

Figure 2.1 Chemical structures of BPA and bisphenolic analytes optimized in this study.

2.2 Chemicals and standards

Bisphenol A (minimum purity 99 %) was purchased from Tokyo Chemical Industry (TCI) (Tokyo, Japan); BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, and BADGE-2HCl were purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile, and methanol, and analytical grade hexane and ethyl acetate were purchased from Labscan Asia Co. Ltd. (Bangkok, Thailand). Stock standard solutions were individually prepared in acetonitrile, and kept in the refrigerator for not more than three months. All working standard solutions were freshly prepared prior to use. Mobile phases were prepared using HPLC grade acetonitrile and HPLC grade

water. Quantitative results were obtained by comparison against external working standards and calibration curves were plotted in the range of 100 µg/L to 2000 µg/L. Oasis HLB cartridges (200 mg, 6 cc) were purchased from Waters (Milford, MA, USA) for solid phase extraction.

2.3 Apparatus

HPLC analyses were performed using a Waters 2695 separations module and a Waters 2475 fluorescence detector (Milford, MA, USA); excitation wavelength: 235 nm; emission wavelength: 317 nm; injection volume: 10 µL; run length: 42 min; column temperature: 25 ^{o}C; column: Nucleosil-100, 5 µm, C18, 250 mm x 4 mm i.d (Hichrom Limited- Berkshire, UK); flow rate: 0.4 mL per minute, where 0 – 1 min: 40: 60 A: B (v/v); 22 min: 50: 50 A: B (v/v); 35.5 – 36 min: 95: 5 A: B (v/v); 38.5 – 42 min: 40:60 A: B (v/v), where A = acetonitrile, and B = HPLC grade water. Prior to each series of chromatographic separations, the analytical column was conditioned for 30 min with methanol, and equilibrated with (40: 60, v/v) acetonitrile: water for 42 min to provide a stable baseline for subsequent chromatographic analysis. Ten minutes of equilibration was required before the next injection. Method robustness was tested using three different columns: Nucleosil-100 5 µm, C18, 250 mm x 4 mm i.d (Hichrom Limited- Berkshire, UK); Hypurity Elite Hypersil ODS 5 µm, 250 mm x 4 mm i.d (Alltech- Massachusetts, USA); Shim-Pack VP-ODS 5 µm, 250 mm x 4 mm i.d (Shimadzu Corporation- Kyoto, Japan).

2.3.1 HPLC Analysis

During the preliminary investigation, the HPLC analyses of all seven bisphenolic analytes were carried out using gradient elution on a Waters 2695 separations module, with fluorescence detection (Waters 2475 Multi λ Fluorescence Detector); Excitation wavelength: 235 nm; Emission wavelength: 317 nm; Injection volume: 10 µL; Run length: 42 min; Column temperature: 25 ^{o}C; Column: Nucleosil-100, 5 µm, C18, 250 mm x 4 mm i.d (Hichrom Limited); Flow rate: 0.4 mL per minute, where 0 – 1 min: 40: 60 A: B (v/v); 22 min: 50: 50 A: B (v/v); 35.5 – 36 min: 95: 5 A: B (v/v); 38.5 – 42 min: 40:60 A: B (v/v), where A = acetonitrile, and B = filtered, deionised water.

During the migration survey, the HPLC analyses of all eleven bisphenolic analytes were carried out on a Shimadzu HPLC system equipped with a RF-10AXL fluorescence detector; LC-10AD pump, DGU-14A degasser, SIL-10AI auto-injector, CTO-10ACVP column oven, and SCL-10AVP system controller. Excitation wavelength: 235 nm; Emission wavelength: 317 nm; Injection volume: 20 µL; Run length: 95 min; Column temperature: 20 ^{o}C; Column: Hypersil ODS, 5 µm, C18, 250 mm x 4 mm i.d (Hypersil); Flow rate: 0.4 mL /min, where 0 – 3 min: (30: 70 A: B, v/v); 15 min: (35: 65 A: B, v/v); 22 min: (41: 59 A: B, v/v); 85 – 85.5 min: (68: 32 A: B, v/v); 86 - 95 min: (30: 70 A: B, v/v), where A = acetonitrile, and B = filtered, deionised water.

Prior to each series of chromatographic separations, the analytical column was conditioned for 30 min with methanol, and equilibrated with (40: 60, v/v) acetonitrile:

water for 45 min to provide a stable baseline for subsequent chromatographic analysis. Ten minutes of equilibration was required before the next injection.

2.4 Samples

Food analysis proficiency assessment scheme (FAPAS®) test materials for BADGE-2HCl, BADGE-H_2O-HCl, and BADGE-HCl (series T1224) were purchased from the Central Science Laboratory (York, UK). Canned food samples of oily, aqueous or acidic media analyzed in this study were obtained from local supermarkets, and 2 aliquots of each sample were taken for duplicate analyses.

For each analytical run, a sample blank prepared using suitable food simulants, *i.e.*, corn oil, 10 % ethanol, or 3 % acetic acid, and a fortified sample (w/w) of the appropriate food simulant, were prepared to estimate the degree of recovery. In order to ensure accurate analytical results, the food simulants were analyzed separately to ensure that they were free from any interfering contaminants.

2.5 Sample Preparation

2.5.1 Extraction of bisphenolic analytes from food

Extraction - The contents in the canned food sample were homogenized before 40 mL of acetonitrile were added to 5 g of the food sample in a round bottomed flask. The

mixture was shaken in the round bottom flask for 25 min before filtering the contents through a Whatman no. 41 filter paper into a separatory funnel. The round bottomed flask was then rinsed with another 10 mL of acetonitrile into the separatory funnel, and 75 mL of n-hexane were added to the contents in the separatory funnel. The mixture was shaken for 2 min, and the 2 immiscible layers were allowed to separate for 25 min. The acetonitrile layer was removed and retained. The hexane layer was washed twice, first with 30 mL of acetonitrile, then with another 20 mL of acetonitrile. The acetonitrile extracts were combined and the solvent was removed using a rotary evaporator.

Solid phase extraction - Oasis HLB® cartridges (6 cc, 200 mg) were conditioned using 5 mL of methanol, and then equilibrated with 4 mL methanol: water (5:95, v/v). After dissolving the dried samples with 3 mL methanol : water (5:95, v/v), they were loaded onto the cartridges and washed with 4 mL methanol : water (20: 80, v/v). The analytes were eluted with 2 mL of methanol twice, followed by 2 mL of methanol: ethyl acetate (50:50, v/v), and 2 mL of ethyl acetate, into screw-capped glass vials. Following that, the samples were blown dry with a stream of nitrogen, reconstituted with 1 mL of (90: 10, v/v) acetonitrile: water, and filtered using 0.20 µm nylon filters into HPLC vials prior to analysis.

2.6 Optimization of sample extraction method

2.6.1 Liquid-liquid extraction clean-up efficiency

To determine the standing time necessary for optimum extraction efficiency of the liquid-liquid extraction, fortified corn oil samples spiked with all seven analytes at 1000 µg/kg levels, were subjected to a three-time liquid-liquid extraction, during which the standing times of 10, 15, 20, 25, and 30 min were evaluated by measuring the analyte concentrations in the combined acetonitrile extracts by HPLC for their efficiencies. It was found that the samples with standing times of 25 and 30 min resulted in similar high recoveries (Figure 2.2), however, for convenience, 25 min was chosen as the standard extraction time for subsequent analyses.

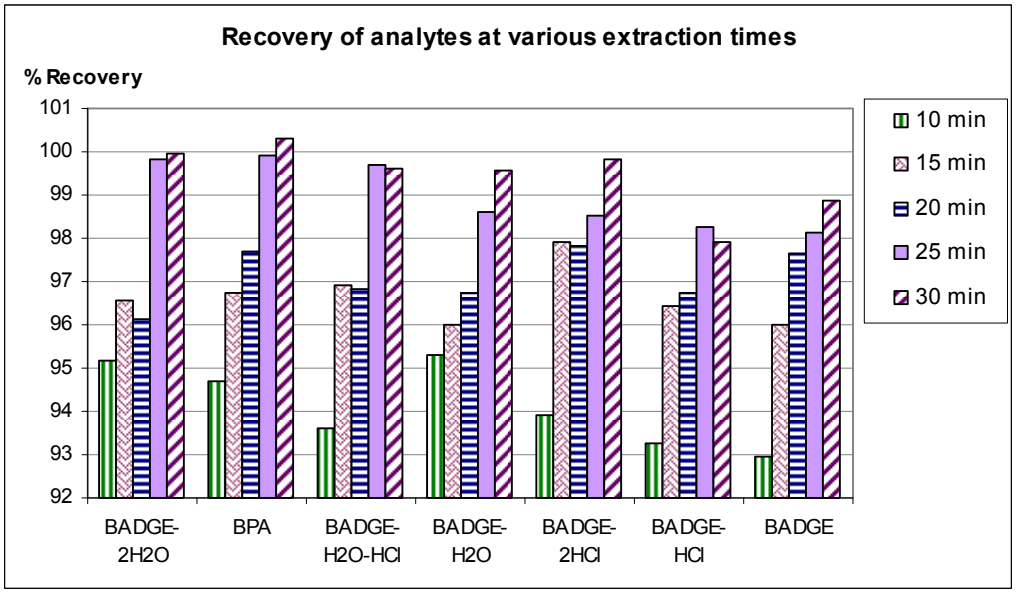

Figure 2.2 Recoveries of liquid-liquid extraction (n = 3) based on different extraction times

2.6.2 Suitability of SPE wash solvent

As the amounts of BPA, BADGE and its derivatives in the food samples were expected to be very low, it was critical to remove as much interfering compounds as possible. Hence, a subsequent clean-up procedure using SPE was deemed necessary.

A series of optimizations on the wash solvent strength using different volume ratios of methanol/water was performed in order to determine the optimum solvent strength for the removal of interfering compounds in the food extracts without compromising on the recovery of the analytes. After conditioning and equilibrating the solid phase extraction cartridges, 1 mL of a mixed standard solution containing all the seven analytes at 1000 µg/L level were loaded onto the cartridges. Wash solvents of differing solvent strengths (10 % methanol solution to 70 % methanol solution) were transferred into the respective cartridges, and the wash eluates were analyzed by HPLC for their analyte content. Results indicated that the 20 % methanol solution had no leaching effect on analyte retention within the SPE cartridge during the washing step (Figure 2.3), hence, this wash solvent was standardized for subsequent analyses. With the additional SPE clean-up procedure using methanol/water, interfering polar compounds are likely to be removed from the food samples.

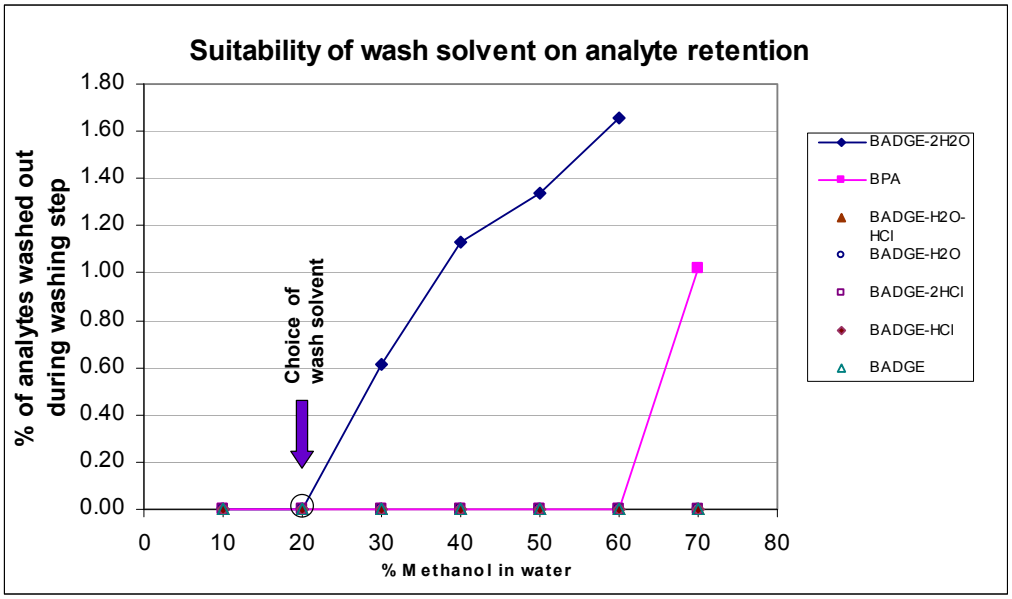

Figure 2.3 Effect of different methanol solutions as SPE wash solvents on analyte retention in the SPE cartridges.

2.6.3 SPE elution solvent efficiency

The use of only one solvent for eluting the analytes was insufficient to bring about satisfactory recoveries as the analytes had a broad range of polarities. Hence, the optimum elution solvent system had to incorporate a series of solvents with gradual decrease in polarities in order to elute all the analytes effectively. After conditioning and equilibrating the solid phase extraction cartridges, 1 mL of a mixed standard solution containing all the seven analytes at 500 µg/L level were loaded onto their respective cartridges (identified as SPE -1 to SPE - 4), washed with 20 % methanolic solution, but eluted using different elution solvent systems. Table 2.1 shows the results of the optimization process for determining the optimum elution solvent system.

Table 2.1 Recoveries of analytes obtained using different types of SPE elution solvents from the optimization process. Analytes that were found below the limit of detection are labeled as ND.

	Percentage recovery of analytes in SPE elution solvent optimization						
	BADGE-$2H_2O$	**BPA**	**BADGE-H_2O-HCl**	**BADGE-H_2O**	**BADGE-2HCl**	**BADGE-HCl**	**BADGE**
SPE -1	89.97	59.74	87.06	58.35	61.92	10.11	ND
SPE -2	93.75	85.94	92.11	85.31	92.21	77.77	19.75
SPE -3	93.02	90.06	94.88	86.25	97.06	89.95	87.56
SPE -4	100.14	94.94	100.67	92.48	99.16	93.38	93.08

The analyte-loaded cartridge SPE -1 was eluted once with 2 mL of methanol, while SPE -2 was eluted twice with 2 mL of methanol. SPE -3 was eluted twice with 2 mL of methanol followed by 2 mL of (50:50, v/v) methanol: ethyl acetate, while SPE -4

was eluted twice with methanol, followed by 2 mL (50:50, v/v) methanol: ethyl acetate, and finally with another 2 mL of ethyl acetate. Clearly, recoveries for all the analytes (92.48 - 100.14 %) were superior in the eluents collected from SPE -4 which had incorporated an additional elution step using a relatively less- polar ethyl acetate organic solvent.

2.6.4 Chromatographic Analysis

The use of gradient elution was necessary to achieve optimum baseline separation for all seven structurally-similar analytes, and the retention times of the various analytes were found to be 9.46, 20.85, 22.43, 23.74, 35.61, 36.47, and 37.37 min for BADGE-2H_2O, BPA, BADGE-H_2O-HCl, BADGE-H_2O, BADGE-2HCl, and BADGE-HCl, and BADGE, respectively (Figure 2.4).

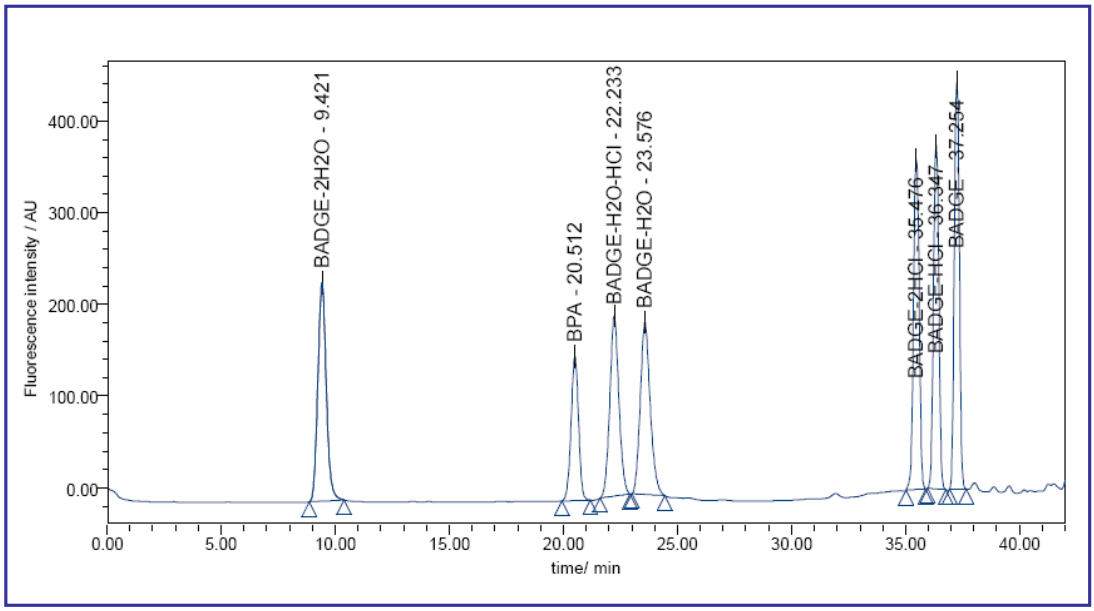

Figure 2.4 Fully resolved chromatographic separation of a standard mixture containing all seven BPA and BADGE analytes at 1500 µg/L level.

2.7 Method Validation

2.7.1 Linearity, LOD and LOQ, and Robustness

Linearity was assessed by inspecting the detection signals as a function of analyte concentration, with the aid of a regression line by the method of least-squares (Table 2.2). The seven analytes were evaluated for linearity using concentration levels of 200, 400, 600, 800, 1000, 1500 and 2000 μg/L, and duplicate injections were made for each concentration level. The correlation coefficient obtained were all $\geq$ 0.999. The limit of detection (LOD) of the analytical procedure is the lowest concentration of analyte that can be measured with definable statistical certainty in a sample, and were calculated from the levels of the various analytes equivalent to three times the standard deviation of noise on analysis, while the limits of quantitation (LOQ) were calculated from the concentration of the analytes that provided signals equal to ten times the signal to noise on analysis. The LODs and the LOQs of the various analytes ranged between 4.5– 7.9 μg/kg, and 13.7 - 24.1 μg/kg, respectively.

The robustness of the analytical method has been established on three different HPLC columns (Nucleosil-100, Hypurity Elite Hypersil ODS and Shim-Pack VP-ODS), and on different HPLC gradient elution programmes. Peak resolutions remained similar despite the different conditions tested.

Table 2.2 Linearity (n=3) and LODs of various bisphenolic analytes determined during the study.

Analyte	**Concentration range / (µg/L)**	**Correlation coefficient (R^2; n =3)**	**LOD of analyte** in food / **(µg/kg)**	**LOQ of analyte** in food / **(µg/kg)**
BADGE-2H2O	200 – 2000	0.9993	6.9	20.9
BADGE-H2O-HCl	200 – 2000	0.9998	4.6	14.0
BPA	200 – 2000	0.9998	4.5	13.7
BADGE-H2O	200 – 2000	0.9998	5.0	15.2
BADGE-2HCl	200 – 2000	0.9995	7.4	22.3
BADGE-HCl	200 – 2000	0.9995	7.2	22.0
BADGE	200 – 2000	0.9994	7.9	24.1

2.7.2 Precision

Precision is the measure of how close results are to one another, and is evaluated by making repetitive measurements for the entire method. Excellent inter-day precision data (n= 10) and intra-day precision data (n=5) were obtained on a 200 µg/kg spiked sample containing all seven analytes. The relative standard deviation (RSD) ranged from 0.20 to 2.96 % for the inter-day precision tests, and 0.04 to 2.82 % for the 3-day intra-day precision tests. The RSD % was then calculated by dividing the standard deviation by the mean, and multiplying the value by 100 %.

2.7.3 Accuracy

The accuracy of the method was assessed at three concentration levels - 200, 1000 and 2000 μg/kg. Ten fortified oil samples at each concentration level were extracted and analyzed using the optimized conditions. Excellent percentage recoveries (86.07 to 114.06 %) were obtained with acceptable variation (RSD: 2.63 to 5.15 %).

2.8 Analysis of Canned Food Samples

The fully optimized and validated analytical method was later applied for the analysis of the levels of BPA and BADGE- related analytes in canned food samples (Table 2.3). Detectable amounts of BPA (0.0328 to 0.1645 mg/kg) were found. However, these concentrations were far below the current specific migration limits of 0.6 mg/kg of food for BPA, 9 mg/kg of food for the sum of BADGE, BADGE-H_2O, and BADGE-$2H_2O$, and 1 mg/kg for the sum of BADGE-HCl, BADGE-2HCl and BADGE-H_2O-HCl, respectively, as imposed by the European Commission [1,2].

The other major contaminants present in the foods were BADGE-$2H_2O$ (detected in 67 % of food samples), BADGE-H_2O-HCl (detected in 50 % of the food samples), and BADGE-2HCl (detected in 34 % of the food samples). Judging from the significantly lower concentrations of the monosubstituted BADGE-related compounds determined in these food samples, it suggests that the monosubstituted BADGE-related compounds may have undergone further hydrolysis within the food matrix to form the more thermodynamically stable disubstituted BADGE-related

compounds [7]. BADGE was detected in only four samples – one aqueous green pea sample, and three oily meat samples. The oily nature of the three meat samples may have reduced the rate of BADGE hydrolysis and hydrochlorination reactions. The occurrence of BADGE in the green pea sample may have been due to a combination of factors such as its higher food-to-aqueous content, and a longer length of shelf display time.

Table 2.3 Results of the analysis of various canned foods (n=2). Analytes that were found below the limit of detection were labeled as ND. Fortified samples (w/w) were prepared by pipetting a small volume of stock standard solution into the round bottomed flask, and gently evaporating off the solvent using a stream of nitrogen gas. 5 g of the appropriate food simulant was then weighed into the same vessel for recovery studies using the sample preparation method described.

Type of food	Concentration of analytes in food / (mg/kg)							Age of canned food from manufacture date / months
	BPA	BADGE-$2H_2O$	BADGE-H_2O	BADGE-H_2O-HCl	BADGE-2HCl	BADGE-HCl	BADGE	
Young Corn in Brine	0.07	0.40	ND	0.08	ND	ND	ND	11
Braised Peanuts	0.10	0.12	ND	ND	ND	ND	ND	9
Mushrooms	0.04	0.12	ND	ND	ND	ND	ND	12
Baked Beans	0.04	0.09	ND	ND	< LOQ	ND	ND	10
Green Peas	0.05	0.10	ND	0.04	ND	ND	0.11	11
Honey sea coconut	0.04	ND	ND	ND	ND	ND	ND	10
Sliced Mango	0.16	0.10	ND	ND	ND	ND	ND	20
Pineapple slices	0.03	0.10	ND	0.20	0.17	ND	ND	16
Pork Luncheon Meat	0.14	ND	0.03	0.04	ND	ND	0.05	8
Stewed Pork	0.05	0.08	ND	0.03	ND	ND	ND	13
Spiced Pork Cubes	0.04	ND	0.05	0.04	0.81	< LOQ	0.05	4
Sandwich Tuna	0.11	ND	0.03	ND	0.08	ND	0.44	27
Sample blank	ND	ND	ND	ND	ND	ND	ND	-

Percentage recovery of analytes in fortified food simulants (w/w)

	BPA	BADGE-$2H_2O$	BADGE-H_2O	BADGE-H_2O-HCl	BADGE-2HCl	BADGE-HCl	BADGE
Oily food simulant	105.2	94.5	89.9	93.6	99.8	94.0	90.0
Aqueous food simulant	90.2	92.5	88.6	89.9	91.9	87.3	88.5
Acidic food stimulant	87.3	92.2	91.8	96.9	103.9	93.1	87.4

The analysis of the food samples were performed together with fortified food simulants to assess the recovery of the analytical procedure using recommended standard food simulants [8]. The mean values obtained from the FAPAS test material analysis performed in duplicate were 453.63 µg/kg for BADGE-HCl-H_2O; 428.76 µg/kg for BADGE-2HCl; and 76.07 µg/kg for BADGE-HCl (CV 0.34 %, 1.05 % and 3.54 %, respectively). These values were in agreement with the assigned values of 491 µg/kg for BADGE-HCl-H_2O; 477 µg/kg for BADGE-2HCl; and 90 µg/kg for BADGE-HCl.

2.9 Conclusions

The developed method for the simultaneous determination of BPA, BADGE, BADGE-H_2O, BADGE-2H_2O, BADGE-H_2O-HCl, BADGE-HCl, and BADGE-2HCl has been shown to be a more suitable method for the extraction and separation of the various bisphenolic analytes at low limits of detection. More importantly, the method can be applied to a wide range of food, which is vital for analyzing the broad range of complex food matrices for the undesirable food contaminants. Quantitative results indicated that the levels of BPA, BADGE, and all the hydrolysed and hydrochlorinated derivatives of BADGE detected in the variety of canned foods tested were below the specific migration limits imposed by the European Commission [1,2].

2.10 References

[1] Official Journal of the European Union (19 November 2005) No. L 302/28, Commission Regulation (EC) No 1895/2005.

[2] Official Journal of the European Union (10 March 2004) No. L 71/8, Commission Directive 2004/19/EC.

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CHAPTER 3

Chapter 3

Simultaneous determination of Bisphenol A, Bisphenol F, Bisphenol A Diglycidyl Ether and its derivatives, and Bisphenol F Diglycidyl Ether and its derivatives from Can Substrates into Canned Foods using Reversed Phase- High Performance Liquid Chromatography with Fluorescence Detection

3.1 Introduction

With respect to the toxicological issues relating to the bisphenolic analytes used in can coatings, the Commission Directive 2002/16/EC was imposed to lay down specific migration limits on the use of bisphenol A diglycidyl ether (BADGE) and its derivatives, as well as Bisphenol F diglycidyl ether (BFDGE) and novolac glycidyl ethers (NOGE) and some of their derivatives in certain epoxy derivatives in materials and articles intended to come into contact with food, with the intention of avoiding risks to human health and barriers to free movement of goods. The Directive stated the use of BADGE and/or BFDGE/NOGE may only be continued till 31 December 2004, and that BADGE was given a transitional period till 31 December 2005, in view of the expected submission of new toxicological data and evaluation by the European Food Safety Authority. With the subsequent new toxicological data submitted, the specific migration limits (SML) were revised: for BADGE, BADGE-H_2O, and BADGE-$2H_2O$, the sum of migration of migration cannot exceed 9 mg/kg in food or food simulants, or 9 mg/6 dm^2 in the food packaging material. Similarly, for

BADGE-HCl, BADGE-H2O-HCl and BADGE-2HCl, the sum of migration of migration cannot exceed 1 mg/kg in food or food simulants, or 1 mg/ 6 dm^2. In addition to these, the use and presence of BFDGE and NOGE can no longer be permitted as from 1 January 2005 onwards, although the depletion of existing stocks should be allowable [1].

In consideration of the revised Directives of the European Commission, the objectives of this part of the research was to develop a method for simultaneously determining these bisphenolic analytes: BPA and BPF, together with the wide range of BADGE, BFDGE and their derivatives (BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE-$2H_2O$, and BFDGE-2HCl) in both can coatings and food using reversed-phase HPLC with fluorescence detection, which have not been fully covered by other research groups to date. From the literature available, the utilization of reverse-phase high performance liquid chromatography (RP-HPLC) combined with fluorescence detection has been the most common analytical technique employed for the determination of BPA in food [2-5]. Bisphenol A has also been analyzed after extraction by the use of gas chromatography- mass spectrometry (GC-MS) [6,7]. The analytes BADGE, BFDGE and some of their hydrolysis and hydrochlorinated products have also been analyzed using RP-HPLC by some research groups [8-12]. Other analytical techniques also include the use of normal-phase HPLC [13] and LC-MS [14-17].

In this part of the research, the content of the bisphenolic analytes from a range of seafood-based and meat-based food products was also investigated; solid-food portions and liquid-food portions within the same sample were analyzed individually

where possible, to achieve a clearer understanding of the migration phenomenon. For the first time, the profile of bisphenolic analytes present in both can coatings and food was also compared and contrasted, so that the effect of the food matrix on migration could be better appreciated. The analytical method that was developed was also validated to ensure that the reported method was fit for its intended purpose; therefore, relevant method validation studies is also presented and discussed in the appropriate sections.

The other BFDGE-related analytes, namely, BFDGE-H_2O, BFDGE-HCl and BFDGE-H_2O-HCl were not commercially available at the time of study, therefore, they were not included in this analysis.

3.2 Chemicals and standards

Bisphenol A (minimum purity 99 %) was purchased from TCI (Tokyo, Japan); Bisphenol F, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE, BFDGE-$2H_2O$, and BFDGE-2HCl were purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile and methanol; analytical grade hexane, ethyl acetate and dichloromethane were purchased from Labscan Asia (Bangkok, Thailand).

Stock standard solutions were individually prepared in acetonitrile, and kept in the refrigerator for not more than three months. All working standard solutions were freshly prepared prior to use. Mobile phases were prepared using HPLC grade acetonitrile and HPLC grade water. Quantitative results were obtained by comparison

against external working standards and calibration curves were plotted in the range of 100 to 2000 µg/L. Oasis HLB cartridges (200 mg, 6 cc) were purchased from Waters (Milford, Massachusetts, USA) for solid phase extraction.

3.3 Apparatus

HPLC analyses of all eleven bisphenolic analytes were carried out on a Shimadzu HPLC system (Tokyo, Japan) equipped with a RF-10AXL fluorescence detector. Excitation wavelength: 235 nm; emission wavelength: 317 nm; injection volume: 20 µL; run length: 95 min; column temperature: 20 ^{o}C; column: Hypurity Elite Hypersil ODS, 5 µm, C18, 250 mm x 4 mm i.d (Alltech, Massachusetts, USA); flow rate: 0.4 ml /min, where 0 – 3 min: (30: 70 A: B, v/v); 15 min: (35: 65 A: B, v/v); 22 min: (41: 59 A: B, v/v); 85 – 85.5 min: (68: 32 A: B, v/v); 86 - 95 min: (30: 70 A: B, v/v), where A = acetonitrile, and B = HPLC grade water. Prior to each series of chromatographic separations, the analytical column was conditioned for 30 min with methanol, and equilibrated with (30: 70 v/v) acetonitrile: water for 30 min to provide a stable baseline for subsequent chromatographic analysis. Method robustness was tested using two different columns: Hypurity Elite Hypersil ODS 5 µm, 250 mm x 4 mm i.d (Alltech- Massachusetts, USA) and Shim-Pack VP-ODS 5 µm, 250 mm x 4 mm i.d (Shimadzu Corporation- Kyoto, Japan).

3.4 Samples

Thirty five types of can meat and seafood samples available were purchased from the local supermarkets for analysis, and grouped into 2 broad categories, namely, meat-based products and seafood-based products (labeled S1 – S35 in Table 3.1). Descriptions of the 35 samples are appended (Appendix I). Five cans bearing the same batch number were obtained if the individual can weighed more than 200 g; ten cans bearing the same batch number were obtained if the individual can weighed less than 200 g. Awaiting analysis, samples were stored at room temperature (20 °C). To overcome the apparent inhomogeneity of the samples, three to eight cans of the each sample, depending on their net weight, were taken and homogenized using a blender for the required analyses (Table 3.1). The remaining empty cans were then gently rinsed with mild detergent, air-dried, and sorted into can tops, can walls and can bottoms for their residual analyte determination.

Table 3.1 Summary of can food samples analysed in the study.

Markings of Meat Samples	No. of cans used for food analysis	Net weight / g	Age of can food during analysis / months	Markings of Seafood Samples	No. of cans used for food analysis	Net weight / g	Age of can food during analysis / months
S 1	4	454	unknown	S 3	4	425	3
S 2	8	184	unknown	S 8	5	185	2
S 4	4	397	3	S 9	3	425	1
S 5	3	415	3	S 10	4	210	8
S 6	3	420	3	S 11	3	425	7
S 7	3	415	4	S 28	5	185	3
S 12	4	305	3	S 32	3	425	2
S 13	4	533	3	S 33	6	155	2
S 14	4	340	3	S 34	6	190	1
S 15	4	533	8	S 35	6	155	7
S 16	4	298	5				
S 17	4	409	4				
S 18	4	370	3				
S 19	4	340	2				
S 20	4	305	6				
S 21	4	397	5				
S 22	4	397	4				
S 23	3	425	7				
S 24	8	180	9				
S 25	4	256	3				
S 26	8	142	4				
S 27	4	256	5				
S 29	3	320	5				
S 30	3	340	2				
S 31	3	340	3				

To ensure on the quality of the results, a food analysis performance assessment scheme (FAPAS®) test material for BADGE and BFDGE (series T1226) were purchased from the Central Science Laboratory (York, UK) to perform additional validation studies. In order to assess the recovery of the analytical procedure, the

analysis of the food samples in each analytical run were carried out together with fortified food simulants recommended by the US Food and Drug Administration [18]. The fortified sample was prepared by pipetting a known volume of each stock standard solution into a round bottomed flask, and gently evaporating off the solvent using a stream of nitrogen gas. Five grams of the appropriate food simulant werethen weighed into the same vessel for recovery studies using the sample preparation method described in an earlier published paper [19]. The food simulants were also analyzed separately to ensure that they were free from any interfering contaminants. In addition, a blank sample was run alongside the entire analytical procedure to inspect for any contamination that may occur during the course of sample preparation.

3.5 Sample Preparation

3.5.1 Extraction of residual bisphenolic analytes from can lacquer

The can metal substrates were calculated for their total internal surface area, cut into smaller pieces of approximately 2 cm by 2 cm using a pair of tin snips, and soaked in a glass bottle containing 250 mL dichloromethane for 24 hours, following which the contents were stirred carefully to homogenize the contents totally. Eight millilitres of the can residue extracts were transferred into a glass vial, evaporated to dryness, reconstituted with 1 mL of (90: 10, v/v) acetonitrile: water. The samples were filtered using 0.20 μm nylon filters into HPLC vials prior to analysis.

3.5.2 Separation of solid and liquid portions in can food

The food samples containing both solid and liquid portions were separated using a strainer measuring 6 cm in diameter. The unopened can food was well shaken for three minutes on a rotatory platform before carefully opened with a can opener. The total contents in the can were carefully poured through the strainer into a labeled glass bottle, such that the solid portion remains onto the strainer. The remaining solid portion was then transferred to another labeled bottle for subsequent procedure(s).

3.5.3 Determination of bisphenolic analytes in can food

The food samples were extracted using the validated sample extraction method described in Chapter 2 utilizing liquid-liquid extraction and solid phase extraction for sample clean-up prior to HPLC analysis for only BPA, BADGE, and BADGE-derivatives present in food samples [2].

Extraction - The contents in the can food sample were homogenized before 40 mL of acetonitrile were added to 5 g of the food sample in a round bottom flask. The mixture was shaken in the round bottomed flask for 25 min before filtering the contents through a Whatman no. 41 filter paper into a separatory funnel. The round bottom flask was then rinsed with another 10 mL of acetonitrile into the separatory funnel, and 75 mL of n-hexane was added to the contents in the separatory funnel. The mixture was shaken for 2 min, and the 2 immiscible layers were allowed to separate for 25 min. The acetonitrile layer was removed and retained. The hexane layer was

washed twice, first with 30 mL of acetonitrile, then with another 20 mL of acetonitrile. The acetonitrile extracts were combined and the solvent was removed using a rotary evaporator.

Solid phase extraction - Oasis HLB® cartridges (6 cc, 200 mg) were conditioned using 5 mL of methanol, and then equilibrated with 4 mL methanol: water (5:95, v/v). After dissolving the dried samples with 3 mL methanol : water (5:95, v/v), they were loaded onto the cartridges and washed with 4 mL methanol : water (20: 80, v/v). The analytes were eluted with 2 mL of methanol twice, followed by 2 mL of methanol: ethyl acetate (50:50, v/v), and 2 mL of ethyl acetate, into screw-capped glass vials. Following that, the samples were blown dry with a stream of nitrogen, reconstituted with 1 mL of (90: 10, v/v) acetonitrile: water, and filtered using 0.20 µm nylon filters into HPLC vials prior to analysis.

Prior to each series of chromatographic separations, the analytical column was conditioned for 30 min with methanol, and equilibrated with (40: 60, v/v) acetonitrile: water for 45 min to provide a stable baseline for subsequent chromatographic analysis. Equilibration for ten minutes was required before the next injection. The optimized chromatographic separation of the various analytes is shown in Figure 3.1.

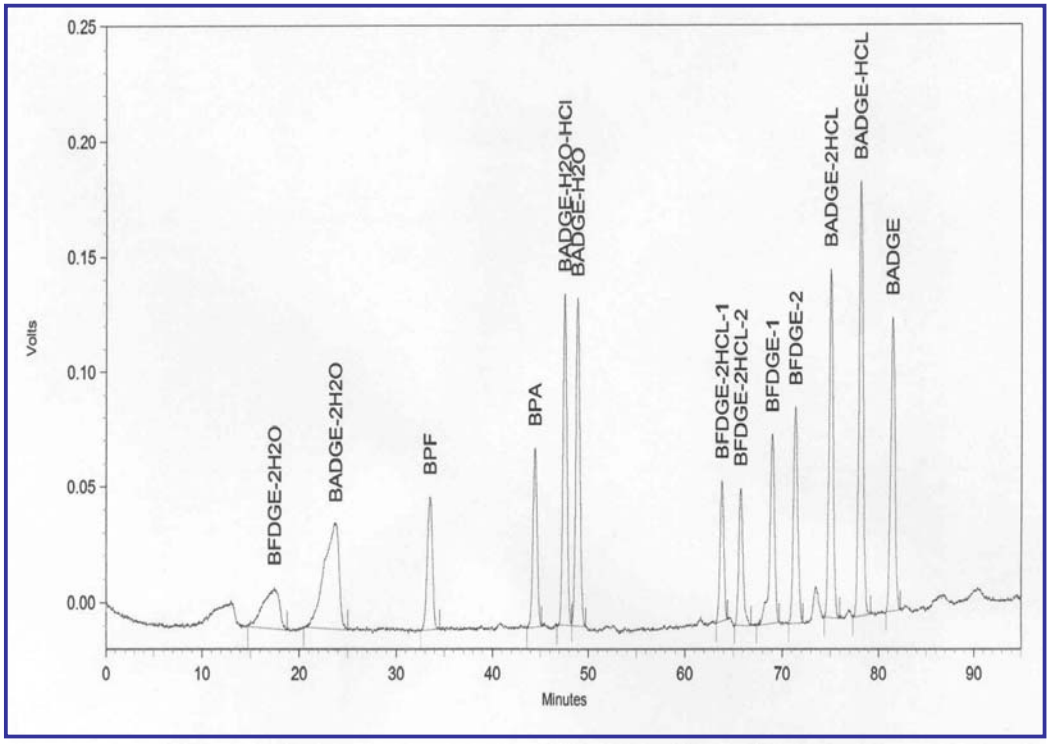

Figure 3.1 HPLC Chromatogram of a 400 µg/L mixed standard solution using fluorescence detection

3.6 Method Validation

3.6.1 Linearity, Range, LOD and LOQ, and Robustness

Linearity was assessed by inspecting the detection signals as a function of analyte concentration, with the aid of a regression line by the least-squares method. The eleven analytes were evaluated for linearity using concentration levels of 100, 200, 400, 800, 1000, 1500 and 2000 µg/L, and the correlation coefficients obtained were all $\geq$ 0.999. The limit of detection (LOD) of the analytical method was calculated from the levels of the various analytes that provided signals equivalent to three times the standard deviation of noise on analysis, while the limits of quantitation (LOQ)

were calculated from the concentration of the analytes that provided signals equal to ten times the signal to noise on analysis. The linearity of the analytes, as well as their respective LODs and the LOQs in food are provided in Table 3.2. These values are conservative estimates and are varied over the course of the project. The robustness of the analytical method has been established on two different HPLC columns (Hypurity Elite Hypersil ODS and Shim-Pack VP-ODS), and on different HPLC gradient elution programmes. Satisfactory peak resolutions were obtained despite the different conditions tested.

Table 3.2 Retention times, correlation coefficient, LOD, and LOQ of the individual analytes in their respective concentration ranges.

Analytes	Retention time in chromatogram/ min	LOD of analyte in food (µg/kg)	LOQ of analyte in food (µg/kg)	Concentration Range/ (µg/L)	Linear Correlation
Bisphenol A (BPA)	44.48	10.00	30.0	100 - 2000	0.9999
Bisphenol A diglycidyl ether (BADGE)	81.50	10.00	30.0	100 - 2000	0.9998
BADGE-H_2O	48.93	10.00	30.0	100 - 2000	0.9999
BADGE-HCl	78.18	10.00	30.0	100 - 2000	0.9999
BADGE-H_2O-HCl	47.58	10.00	30.0	100 - 2000	0.9999
BADGE-2HCl	75.07	15.00	45.0	100 - 2000	0.9996
BADGE-$2H_2O$	23.77	10.00	30.0	100 - 2000	0.9999
Bisphenol F (BPF)	33.53	10.00	30.0	100 - 2000	0.9999
Bisphenol F diglycidyl ether (BFDGE) -1	69.02	10.00	30.0	100 - 2000	0.9998
Bisphenol F diglycidyl ether (BFDGE)-2	71.41	15.00	45.0	100 - 2000	0.9997
BFDGE-2HCl-1	63.83	10.00	30.0	100 - 2000	0.9998
BFDGE-2HCl-2	65.76	10.00	30.0	100 - 2000	0.9999
BFDGE-$2H_2O$	17.47	15.00	45.0	100 - 2000	0.9996

3.6.2 Precision and Accuracy

Precision was assessed by analyzing a 100 µg/L standard solution containing all eleven analytes using the optimized method on 3 consecutive days, with 8 repetitions performed each day, and the RSD % was then calculated by dividing the standard deviation by the mean, and multiplying the value by 100 % (Table 3.3). The intra-day precision was assessed by analyzing a 100 µg/L standard solution containing all eleven analytes using the optimized method every 2 hours for 3 consecutive days, with 5 injections per day. The RSD ranged from 0.57 to 6.95 % for the inter-day precision tests, and 0.65 to 4.90 % for the intra-day precision tests. The precision results are also illustrated in Figure 3.2 for better clarity.

Table 3.3 Recovery studies (n = 10) at 100, 500, and 2000 µg/kg level using fortified oil samples; interday precision results (n =8) and intraday precision results (n =5) results using a 100 µg/L mixed standard solution containing all bisphenolic analytes

	Accuracy Results (n = 10) [RSD % in brackets]			Interday Precision Results (n = 8) [RSD % in brackets]			Intraday Precision Results (n = 5) [SD in brackets]		
	100 µg/kg level	500 µg/kg level	2000 µg/kg level	Concentration / (µg/L)			Concentration / (µg/L)		
Analytes	Mean Recovery / %	Mean Recovery / %	Mean Recovery / %	Day 1	Day 2	Day 3	Day 1	Day 2	Day 3
BADGE-2H_2O	98.48 (2.74)	91.36 (5.39)	92.21 (3.14)	99.39 (3.13)	98.89 (1.50)	99.62 (3.28)	98.53 (3.58)	98.32 (1.51)	100.26 (3.84)
BPA	97.93 (6.50)	97.28 (3.45)	99.75 (2.27)	98.63 (1.54)	97.70 (1.88)	98.43 (2.48)	98.85 (1.74)	98.34 (1.61)	99.62 (1.42)
BADGE-H_2O-HCl	98.39 (3.63)	89.97 (2.81)	96.20 (1.07)	99.39 (1.92)	98.05 (1.76)	95.89 (3.46)	99.32 (2.12)	98.70 (1.76)	96.59 (3.79)
BADGE-H_2O	93.13 (1.93)	87.48 (8.75)	96.08 (1.12)	99.24 (1.56)	98.84 (1.62)	98.79 (1.35)	99.05 (1.60)	99.21 (1.99)	99.38 (1.32)
BADGE-2HCl	93.46 (2.27)	94.25 (8.47)	91.78 (1.13)	101.69 (2.79)	101.20 (3.70)	98.46 (3.54)	101.31 (3.33)	102.20 (2.42)	97.41 (4.19)
BADGE-HCl	97.05 (4.36)	90.11 (4.01)	91.96 (1.19)	96.83 (1.55)	96.944 (1.62)	97.49 (3.07)	96.42 (1.47)	96.92 (1.88)	98.00 (2.53)
BADGE	97.06 (2.34)	93.59 (5.31)	98.27 (0.91)	99.63 (0.75)	100.25 (1.03)	97.45 (3.24)	99.57 (0.84)	100.33 (1.05)	98.73 (3.01)
BFDGE-2H_2O	99.25 (3.35)	89.41 (2.38)	96.98 (3.38)	98.36 (2.78)	97.09 (4.45)	96.32 (6.95)	98.03 (3.52)	98.44 (4.90)	98.44 (3.40)
BPF	97.58 (3.42)	95.49 (3.37)	100.84 (1.29)	99.26 (1.49)	97.28 (2.10)	98.94 (1.11)	99.43 (1.47)	97.98 (2.01)	98.82 (1.09)
BFDGE-2HCl	92.72 (2.20)	94.83 (3.24)	99.59 (1.29)	98.51 (3.25)	98.13 (0.57)	98.04 (2.78)	98.92 (3.93)	98.18 (0.65)	96.75 (2.11)
BFDGE	100.49 (5.33)	97.21 (3.09)	99.97 (2.49)	93.82 (4.38)	95.08 (2.40)	96.27 (2.33)	94.37 (3.30)	95.88 (1.41)	96.42 (1.91)

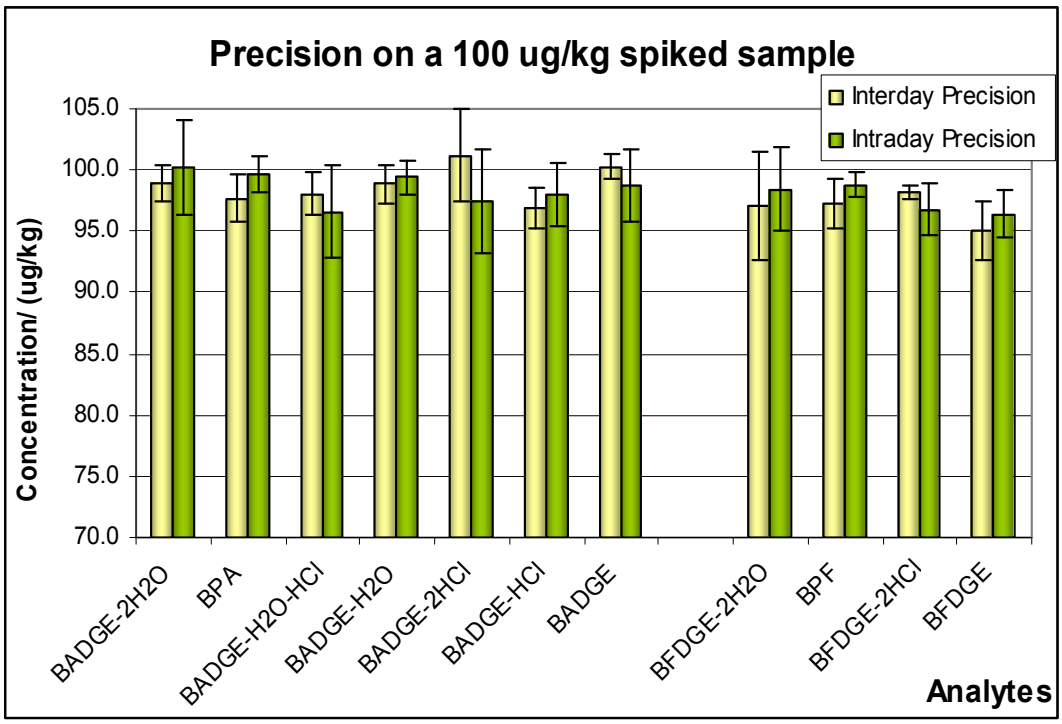

Figure 3.2 Graphical presentation of the precision results of analytes (100 µg/kg), n = 8; Linearity : 100– 2000 µg/L

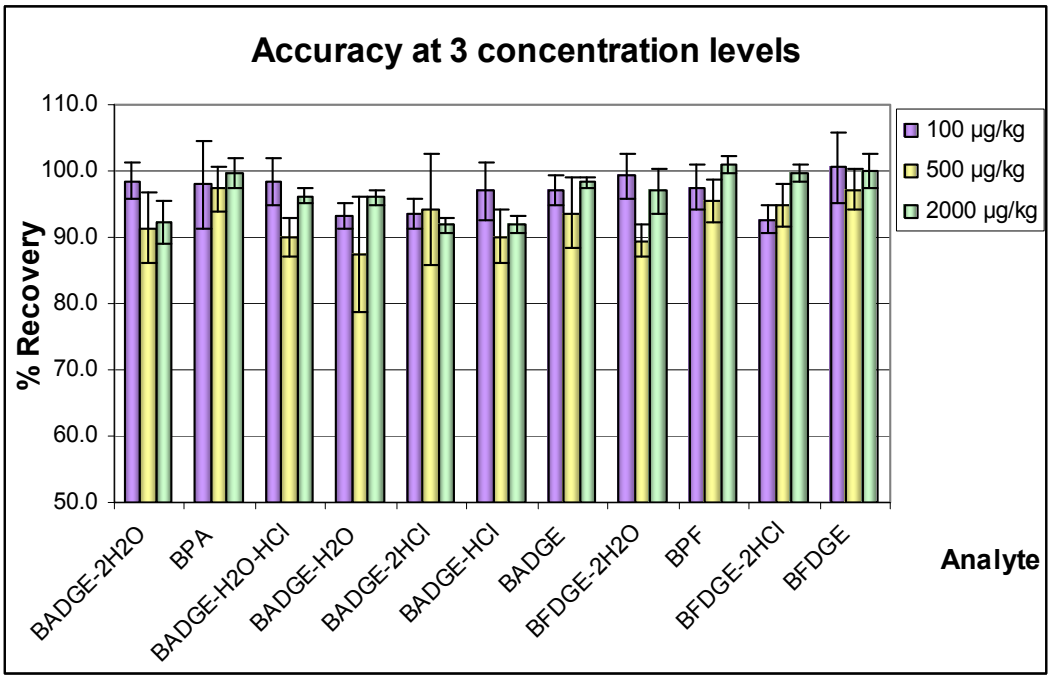

Figure 3.3 Accuracy results on 100, 500 and 2000 µg/kg spiked samples (n = 10)

The accuracy of the method was assessed at three concentration levels - 100, 500 and 2000 µg/kg. Corn oil – a fatty food stimulant, being a more complicated matrix compared to the aqueous food stimulant (10 % ethanol solution), or acidic simulant (3 % acetic acid solution), was used for recovery studies. Fortified oil samples were extracted and analyzed, and their recoveries were illustrated (Figure 3.3). Recoveries were satisfactory for all three different concentration levels, ranging from 87.5 to 100.8 % (Table 3.3). The RSD obtained ranged from 0.91 to 8.75 %. To further ensure the quality of the analytical method, a FAPAS® test material was analyzed in duplicate alongside while analyzing the bisphenolic contents in the can food. The mean values obtained were 1483.7 and 947.0 µg/kg for BADGE and BFDGE, respectively (CV 0.04 %; 0.24 %). These values were in very close agreement with the assigned values of 1483 µg/kg for BADGE, and 949 µg/kg for BFDGE, with percentage relative errors of only 0.05 % for BADGE, and 0.21 % for BFDGE.

3.7 Analysis of Can Food Samples

3.7.1 Effect of the oily food matrix on the migration profile of bisphenolic analytes in solid and liquid food portions

From the investigation of the target analytes in both solid and liquid portions of can food, results revealed that 90.9 % of eleven food samples had the majority of the analytes residing

within the solid food portion as compared to the aqueous liquid food portion, since the analytes had higher affinity for the oily food components.

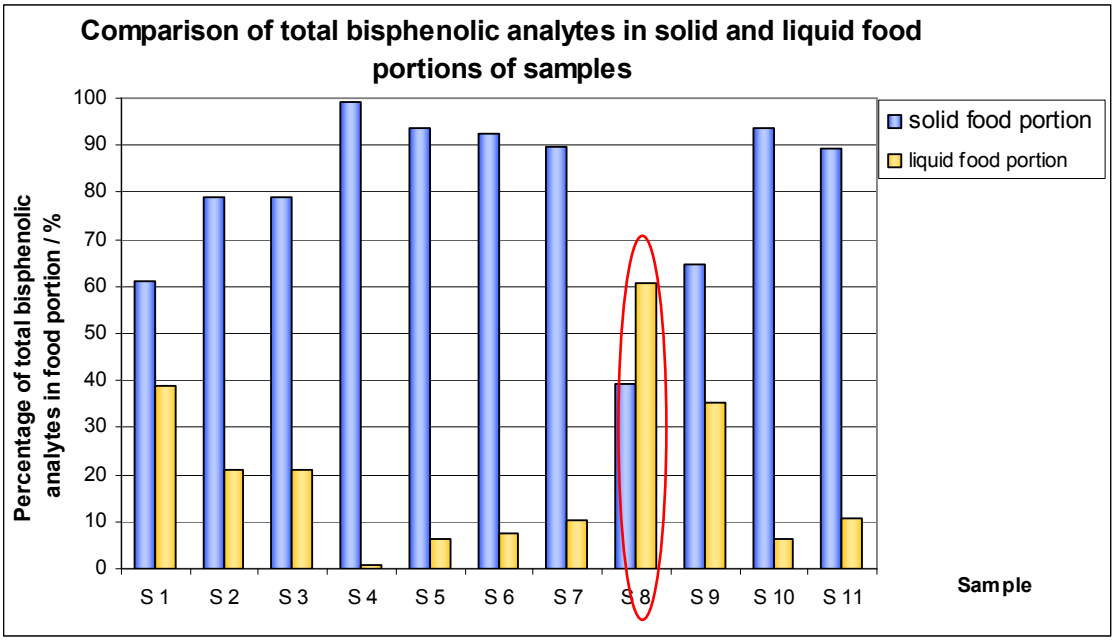

Figure 3.4 Total bisphenolic analyte levels (in percentages) in solid and liquid portions of various food samples.

In the remaining sample S8, the analytes were located preferentially in the oily liquid portion (Figure 3.4 & Table 3.4). Not only that, significantly higher concentrations of BADGE and BFDGE, as compared to the hydrolysis and hydrochlorination products, were also found within the liquid portion in sample S8, where 37.0 % of BADGE was detected as compared to 0.22 % of the hydrolysis products (BADGE-H_2O and BADGE-$2H_2O$) detected, from the sum of all the BPA and BADGE-related analytes in both the solid and liquid portions of the sample.

Table 3.4 Results of BPA and BADGE-based analytes, and BPF and BFDGE-based analytes in S8.

	Content of analytes in sample / (µg/kg)							
	BPA	BADGE	BADGE-H_2O	BADGE-HCl	BADGE-H_2O-HCl	BADGE-2HCl	BADGE-$2H_2O$	Total
solid food	39.01	< LOD	< LOD	23.40	66.17	10.18	312.01	450.77
liquid food	34.83	421.38	2.48	171.05	23.34	45.76	< LOD	698.84

	Content of analytes in sample / (µg/kg)				
	BPF	BFDGE -1	BFDGE-2HCl-1	BFDGE-$2H_2O$	Total
solid food	< LOD	2236.64	< LOD	262.28	2498.92
liquid food	< LOD	8541.03	71.27	35.77	8648.07

Similarly, 76.6 % of BFDGE-1 was found in the oily portion, as compared to 0.32 % of the hydrolysis product (BFDGE-$2H_2O$) from the sum of all the BPF and BFDGE-related analytes in both the solid and liquid portions of the sample. This observation was consistent with the survey findings made by the Food Standards Agency on a variety of 52 types of can fish samples [12], suggesting that the oily environment may have protected BADGE and BFDGE from further hydrolysis and hydrochlorination reactions [13].

3.7.2 Effect of the aqueous food matrix on the migration profile of bisphenolic analytes in solid and liquid food portions

Various trends on the profile of bisphenolic analytes were observed from the analysis of the can, and in foods with higher moisture content. For clarity and for the ease of discussion in

this context, BADGE- based analytes were presented separately from the BFDGE- based analytes. In addition, during the later part of the discussion, the content of analytes in each source (can and food) was summed, and the various classes of compounds (BADGE/ BFDGE, their hydrolysis compounds, and hydrochlorinated compounds) were presented based on percentages of the total bisphenolic content in the respective source. Due to the large sample size taken for this study and the number of analytes determined, selected examples were chosen for a more detailed and effective discussion instead of the usual tabulation of results.

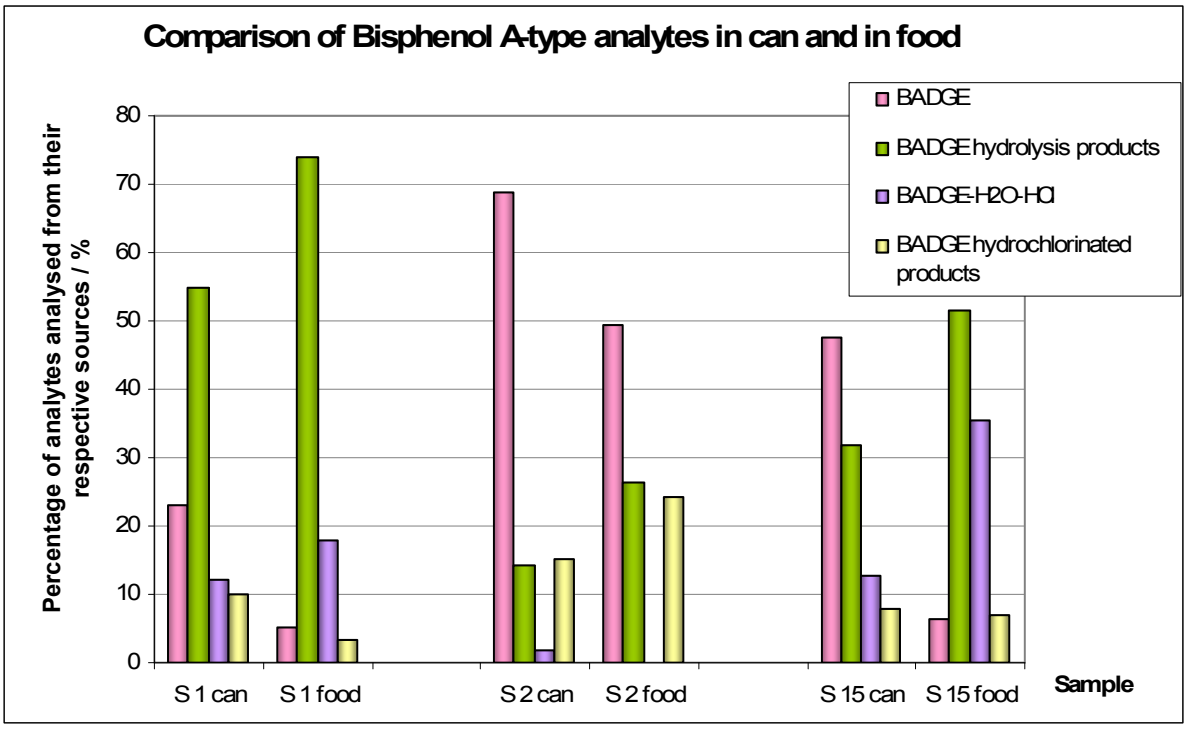

Figure 3.5 Proportions of bisphenol A- type analytes (in percentages) detected in can and food

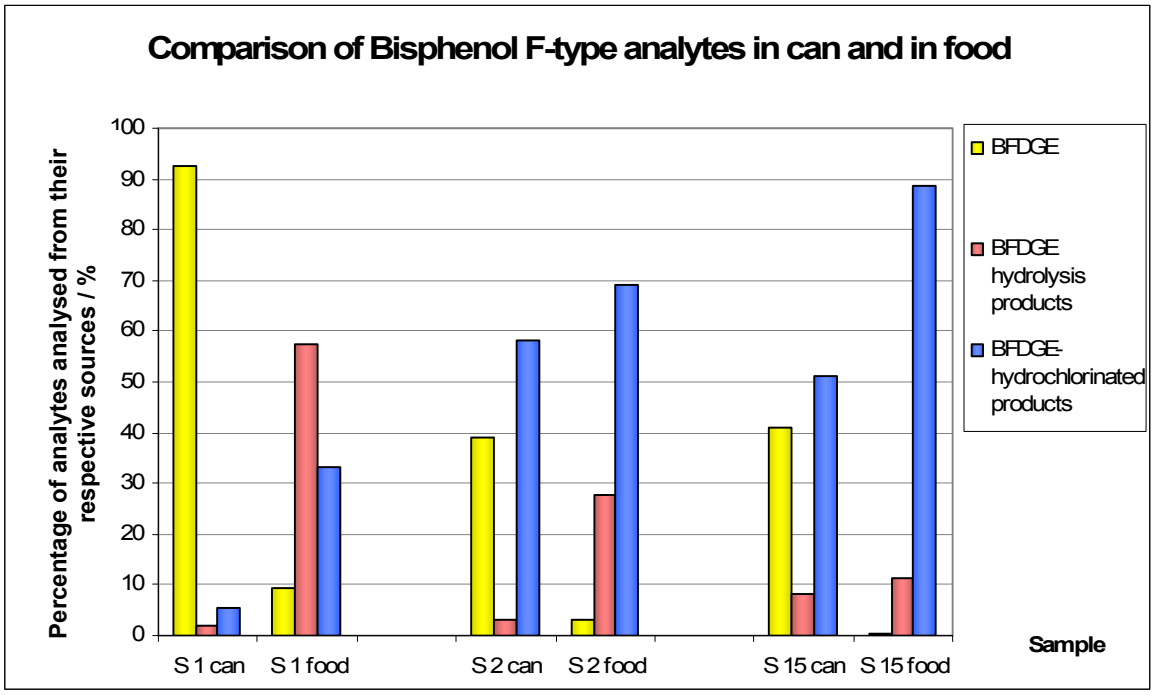

Figure 3.6 Proportions of bisphenol F- type analytes (in percentages) detected in can and food

Figures 3.5 and 3.6 illustrate the various classes of bisphenolic analytes present in the can and food portions of three meat samples (S1, S2 and S15). It was observed that in these three samples, some trends were very distinct, where the can contained relatively higher proportions of BADGE and/or BFDGE, while the analysis performed on the food indicated higher proportions of the hydrolysis and hydrochlorinated bisphenolic compounds present. To further elaborate on this trend, BADGE analysed in the food portion of sample S15 was about 13 % of that found in the can (Figure 3.5). However, the BADGE hydrolysis products increased from 31.7 to 51.4 % in food with respect to the can, while BADGE- H_2O-HCl increased by a factor of 4 from 12.8 % in the can to 51.3 % in food. The BFDGE hydrochlorination products also increased from 50.6 % in can to 88.6 % in food (Figure 3.6).

The presence of higher proportions of hydrolysis and hydrochlorinated bisphenolic compounds in food suggests that reactions must have occurred after the bisphenolic analytes were transferred into food [20]. Hence, the food matrix functioned as an important environment for potential reactions of BADGE and BFDGE, and had an important influence on the major analytes in the sample.

3.8 Conclusions

The simultaneous analysis of BPA, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BPF, BFDGE, BFDGE-$2H_2O$, and BFDGE-2HCl in both can substrates and food has been performed on the range of meat and seafood-based food samples using the validated method. From the results, the presence of the various target bisphenolic analytes in both coatings and in food proved that migration of the contaminants had occurred as a result of the processing conditions and time. Consequently, from the comparative analyses made on both the solid and liquid portions of various can food samples, the bisphenolic analytes were found to have preferential affinity with lipophilic food components. The oily or aqueous food matrix also had important consequences on the bisphenolic migration profile, where the immediate environment may have allowed hydrolysis and/or hydrochlorination reactions of BADGE and/or BFDGE to occur. Overall, it was found that the type of meat had a less significant influence to the migration profile of the bisphenolic analytes determined.

As the specific migration limits of the various bisphenolic compounds have recently been revised by the European Union, the low limits of detection for the various compounds in the reported method enables the enforcement of the various specific migration limits set by the Commission Regulation (EC) No. 1895/2005 [1] and Commission Directive 2004/19/EC [21], and at the same time, utility is provided for both food producers and food quality surveillance institutions.

3.9 References

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CHAPTER 4

Chapter 4

A Fast Determination of Bisphenol A, Bisphenol F, Bisphenol A Diglycidyl Ether and its derivatives, and Bisphenol F Diglycidyl Ether and its derivatives in canned food by Ultra Performance Liquid Chromatography (UPLC ™)

4.1 Introduction

In the previous chapters, the determination of BPA and BADGE analytes, as well as the BPF and BFDGE analytes were discussed thoroughly. These discussions focused on the method optimization, separation and identification of the analytes by classical chromatographic methods, method validation as well as the results from the various studies. However, the analysis time tended to be very time consuming (of longer than 35 min) using the classical reverse phase HPLC methods [1-5] which also utilized significant solvent usage and electricity.

The introduction of the ultra performance liquid chromatograph (UPLC ™) and the development of sub-micron stationary phase particle sizes have brought about exciting developments to the analytical testing scene, where analytes could be determined by the UPLC at a fraction of the existing run times and with better sensitivities, as compared to the conventional HPLC analyses using stationary phases with particle sizes of 3.0 μm or more. Due to the exciting possibilities of the new instrumentation, we attempt to develop a UPLC method for the BPA, BPF, BADGE and its derivatives, as well as BFDGE and its derivatives

in order to benefit from the shortened run-times and sensitivity improvements for increased laboratory throughput.

This chapter will therefore explore the development of the UPLC sample preparation methodology for the whole range of BPA and BPF, both being monomers of BADGE and BFDGE respectively, together with the rest of BADGE, BFDGE and their derivatives, namely BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE-$2H_2O$, and BFDGE-2HCl. The results of the method validation performed will also be presented in this chapter.

4.2 Experimental

4.2.1 Materials and Reagents

Bisphenol A (minimum purity 99 %) was purchased from TCI (Tokyo, Japan); Bisphenol F, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE, BFDGE-$2H_2O$, and BFDGE-2HCl were purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile and methanol; analytical grade hexane and ethyl acetate were purchased from Labscan Asia (Bangkok, Thailand).

Stock standard solutions were individually prepared in acetonitrile, and kept in the refrigerator for not more than three months. All intermediate and working standard solutions were freshly

prepared prior to use. Quantitative results were obtained by comparison against external working standards and calibration curves were plotted in the range of 25 to 1000 μg/L. Mobile phases were prepared using HPLC grade acetonitrile and water that was filtered using a 0.2 μm membrane filter. Oasis HLB cartridges (200 mg, 6 cc) were purchased from Waters (Milford, Massachusetts, USA) for solid phase extraction.

Stock standard solutions (100 mg/L) were individually prepared in acetonitrile, and kept in the refrigerator for not more than three months. Freshly prepared intermediate stock solutions (5 mg/L) were made during each analysis run, by diluting appropriately from the respective stock standard solution using a mixture of acetonitrile and water (50:50, v/v). All working standard solutions were freshly prepared daily prior to use.

4.3 Apparatus

The analyses were performed using a UPLC system coupled to a fluorescence detector (Waters, Milford, MA, USA). Excitation wavelength: 235 nm; emission wavelength: 317 nm; injection volume: 2.5 μL; run-time: 15 min; Post-run time: 15 min; column temperature: 30 ^{o}C; column: Acquity BEH C18, 1.7 μm, 150 mm x 2.1 mm i.d (Waters); flow rate: 0.3 mL min^{-1}, where 0 – 1 min: (18: 82 A: B, v/v); 14 min: (66.3: 33.7 A: B, v/v); 15 - 17 min: (95: 5 A: B, v/v); 17.1min: (18: 82 A: B, v/v), where A = acetonitrile, and B = HPLC grade water

Prior to each series of chromatographic separations, the analytical column was conditioned for at least 30 min with acetonitrile, and equilibrated with acetonitrile : filtered water (18: 82, v/v) for a duration of at least 15 min to provide a stable baseline for subsequent chromatographic analysis.

4.4 Samples

The fortified samples for accuracy studies were prepared by pipetting a known volume of each stock standard solution into a round bottomed flask, and gently evaporating the solvent using a stream of nitrogen gas. Five grams of the food were then weighed into the same vessel for recovery studies using the sample preparation method described in Chapter 2.

4.5 Sample Preparation

Extraction - The contents in the canned food sample were homogenized before 40 mL of acetonitrile were added to 5 g of the food sample in a round bottomed flask. The mixture was shaken in the round bottom flask for 25 min before filtering the contents through a Whatman no. 41 filter paper into a separatory funnel. The round bottom flask was then rinsed with another 10 mL of acetonitrile into the separatory funnel, and 75 mL of n-hexane was added to the contents in the separatory funnel. The mixture was shaken for 2 min, and the two immiscible layers were allowed to separate for 25 min. The acetonitrile layer was removed and retained. The hexane layer was washed twice - first with 30 mL of acetonitrile, then with

another 20 mL of acetonitrile. The acetonitrile extracts were combined and the solvent was removed using a rotary evaporator.

Solid phase extraction - Oasis HLB® cartridges (6 cc, 200 mg) were conditioned using 5 mL of methanol, and then equilibrated with 4 mL methanol: water (5:95, v/v). After dissolving the dried samples with 3 mL methanol : water (5:95, v/v), they were loaded onto the cartridges and washed with 4 mL methanol : water (20: 80, v/v). The analytes were eluted with 2 mL of methanol twice, followed by 2 mL of methanol: ethyl acetate (50:50, v/v), and 2 mL of ethyl acetate, into screw-capped glass vials. Following that, the samples were blown dry with a stream of nitrogen, reconstituted with 1 mL of (50: 50, v/v) acetonitrile: water, and filtered using 0.20 μm nylon filters into HPLC vials prior to analysis.

4.6 Results and Discussion

4.6.1 Chromatographic Analysis

The chromatographic analysis of the 11 bisphenolic analytes was optimized on the UPLC. As the UPLC incorporated very fine particles (1.7 μm) as the stationary phase, the resolution was not only significantly improved, but the analysis benefited greatly with the reduced run time of 15 minutes (Figure 4.1). In addition, three isomers (ortho-para, ortho-ortho and para-para) of BFDGE could be discriminated from the UPLC separation, unlike the conventional HPLC separation, as illustrated in Figure 3.1 in Chapter 3. The separation of the various bisphenolic analytes are illustrated in Figure 4.1, and the corresponding retention times were tabulated in Table 4.1 for clarity.

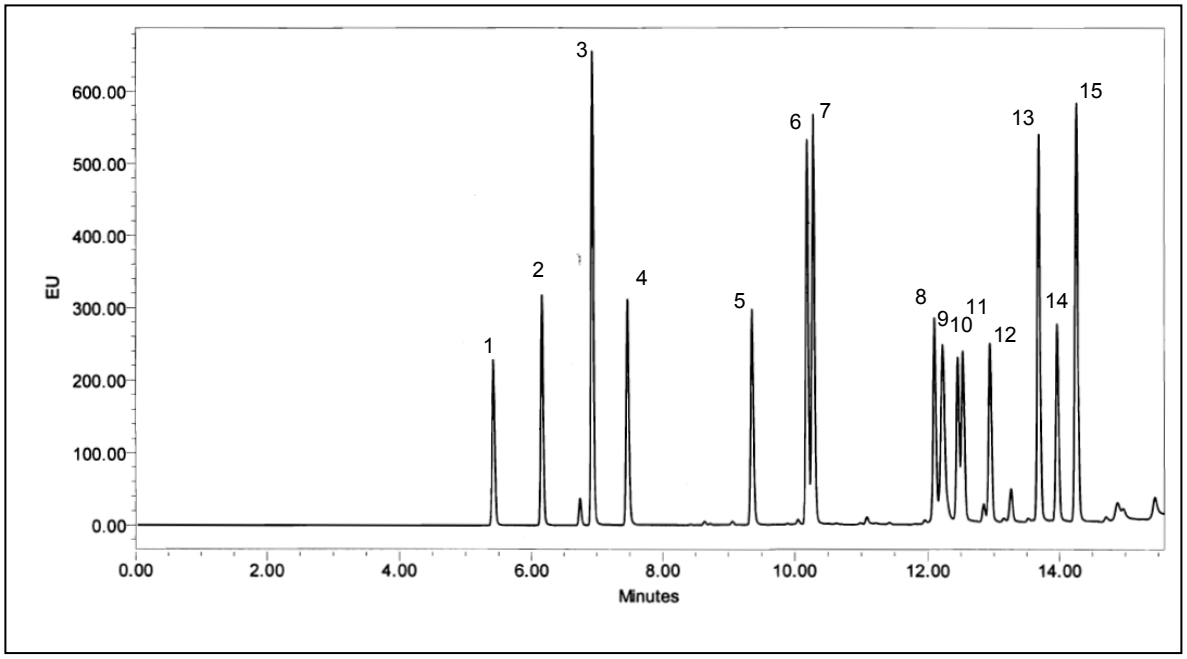

Figure 4.1 Chromatographic separation of the mixture of 11 bisphenolic analytes at 1 mg/L, where 1. BFDGE-2H_2O-1, 2.BFDGE-2H_2O-2, 3. BADGE-2H_2O, 4. BPF, 5. BPA, 6. BADGE-H_2O-HCl, 7. BADGE-H_2O, 8. BFDGE-2HCl-1, 9. BFDGE-1, 10. BFDGE-2, 11. BFDGE-2HCl-2, 12. BFDGE-3, 13. BADGE-2HCl, 14. BADGE-HCl, 15. BADGE.

Table 4.1 Retention times of the bisphenolic analytes analysed using the UPLC. Isomers of the analytes are differentiated by -1; -2 or -3. (i.e. Peak at 12.244 min is the first isomer of BFDGE)

Peak	Retention Time / min	Analyte
1	5.421	BFDGE-2H_2O-1
2	6.159	BFDGE-2H_2O-2
3	6.025	BADGE-2H_2O
4	7.463	BPF
5	9.356	BPA
6	10.184	BADGE-H_2O-HCl
7	10.277	BADGE-H_2O
8	12.100	BFDGE-2HCl-1
9	12.244	BFDGE-1
10	12.451	BFDGE-2
11	12.529	BFDGE-2HCl-2
12	12.939	BFDGE-3
13	13.678	BADGE-2HCl
14	13.961	BADGE-HCl
15	14.253	BADGE

4.6.2 Method Validation

4.6.2.1 Linearity, Range, LOD and LOQ and Robustness

The eleven analytes in the analytical run were evaluated for linearity using concentration levels of 10, 25, 50, 100, 250, 500, and 1000 μg/L. The linear correlation coefficients obtained were all ≥ 0.99. The limit of detection (LOD) of the analytical method, calculated from the levels of the various analytes that provided signals equivalent to three times the standard deviation of noise on analysis, was at 1.5 μg/kg; the limits of quantitation (LOQ),

calculated from the concentration of the analytes that provided signals equal to ten times the signal to noise on analysis, was 4.5 µg/kg. These values are conservative estimates and are varied over the course of the project.

4.6.2.2 Precision and Accuracy

Precision was assessed by analyzing a 25 µg/kg spiked canned baby corn sample containing all eleven analytes using the optimized method with 12 repetitions. The resulting RSD % was then calculated by dividing the standard deviation by the mean, and multiplying the value by 100 %; the results ranged from 0.35 to 3.09 % for the precision tests.

The accuracy of the method was assessed at 50 µg/kg. Six fortified canned mushroom samples were extracted and analyzed using the optimized conditions. Good percentage recoveries ranging from 75.4 to 143.7 % were obtained for the fortified samples at very low fortification levels, with satisfactory variation results (RSD %: 1.37 to 4.07 %).

4.6.3 Quality Assurance

During the development of this method using the UPLC, no suitable available proficiency test materials for bisphenolic analytes were available for sale. Hence, other necessary quality control measures were taken to ensure the quality of the results for the validation of the

method. Samples tested were always analyzed together with a blank, and at least one fortified sample in similar matrices in order to determine the degree of recovery of the sample preparation process. In addition, standard checks were also placed in between the instrumental run to check for any deviations in analyte intensities and retention times with the results of a similar standard of the same concentration analyzed during the beginning of the instrumental run. Results were acceptable only if the blanks were free from interfering contaminants and the retention time shifts of the analytes were less than 5 %.

4.6.4 Improvements to the method

The UPLC method benefited greatly from the reduced run time from 95 min (Fig. 4.2) using the conventional HPLC, as described earlier in Chapter 3, to only 15 min (Fig 4.1). In addition, this method also improved on the sensitivity significantly by more than 3 times. The linear range of the UPLC method was also brought to lower limits to take advantage of the increased sensitivity of the instrument, which allowed the limits of detection of the various analytes to stand at 1.5 μg/kg each, which is a considerable improvement from the previous HPLC method (Table 4.2).

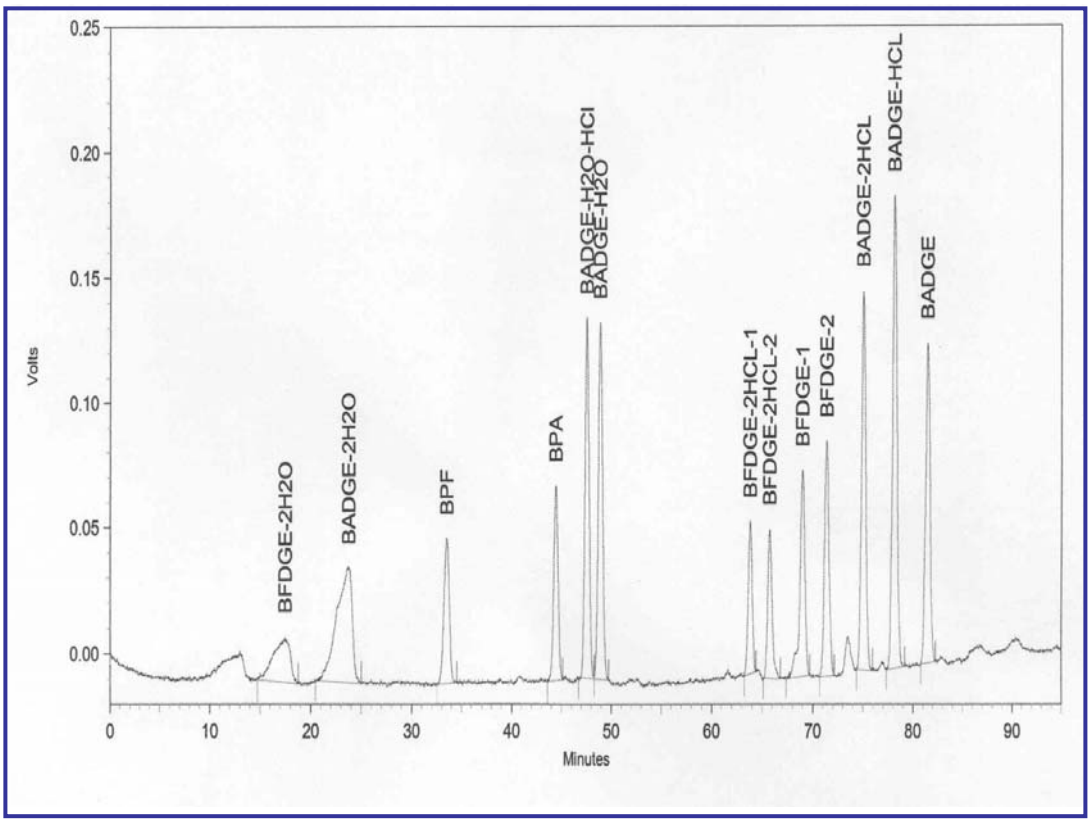

Figure 4.2 HPLC Chromatogram of a 400 µg/L mixed standard solution using conventional HPLC with fluorescence detection.

Table 4.2 Comparison of limits of detection (LOD)s of the various bisphenolic analytes between the UPLC method and the conventional HPLC method.

Analyte	By UPLC		By HPLC	
	LOD of analyte in food (µg/kg)	**LOQ of analyte in food (µg/kg)**	**LOD of analyte in food (µg/kg)**	**LOQ of analyte in food (µg/kg)**
Bisphenol A (BPA)	1.50	4.55	5.00	15.2
Bisphenol A diglycidyl ether (BADGE)	1.50	4.55	5.00	15.2
BADGE-H_2O	1.50	4.55	5.00	15.2
BADGE-HCl	1.50	4.55	5.00	15.2
BADGE-H_2O-HCl	1.50	4.55	5.00	15.2
BADGE-2HCl	1.50	4.55	7.50	22.7
BADGE-$2H_2O$	1.50	4.55	5.00	15.2
Bisphenol F (BPF)	1.50	4.55	5.00	15.2
Bisphenol F diglycidyl ether (BFDGE)	1.50	4.55	7.50	22.7
BFDGE-2HCl	1.50	4.55	5.00	15.2
BFDGE-$2H_2O$	1.50	4.55	7.50	22.7

4.7 Conclusions

The developed method by UPLC presented in this chapter was found to be successful for the simultaneous determination of the eleven bisphenolic analytes in canned food by UPLC, and was shown to be successful in reducing the analysis time significantly by more than 6 times. The method has not only been fully validated, but also found to have an increase in sensitivity by more than 300 %, which brings about an improvement in efficiency, sensitivity and throughput. As a result, savings in terms of the mobile phase cost, run time, and operating costs was been made. This is also the first analytical method that is capable of determining the

eleven bisphenolic analytes simultaneously by the ultra performance liquid chromatography that is fit for its intended use on canned food products.

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CHAPTER 5

Chapter 5

A Specific Method for the Simultaneous Determination of Bisphenol A, Bisphenol F, Bisphenol A Diglycidyl Ether and its derivatives, and Bisphenol F Diglycidyl Ether and its derivatives in Canned Beverages by Positive and Negative ESI- Liquid Chromatography-Tandem MS

5.1 Introduction

The determination of BPA in food has been commonly reported using reverse-phase high performance liquid chromatography (RP-HPLC) combined with fluorescence detection [1-6], as well as gas chromatography- mass spectrometry (GC-MS) [7, 8]. The analytes BADGE, BFDGE and some of their hydrolysis and hydrochlorinated products have also been reported to be analyzed using RP-HPLC [9-15]. Other analytical techniques available in the literature also included the use of normal-phase HPLC [16] and LC-MS [17-21]. However, there are few reported methods of LC tandem MS (LC-MS/MS) analysis of these bisphenolic compounds in the literature, and this provides an area for the authors to explore, in terms of developing a selective and specific method for the confirmation and quantitation of all these bisphenolic analytes, especially in complicated food matrices, where interferences may occur to hinder accurate chromatographic identification and quantitation, and the confirmation of the presence of the analytes by their mass-to-charge ratios would be most suitable. An example of this method's confirmatory ability will be illustrated on the in-house prepared canned coffee drink. To date, no other research groups have reported multi-reaction monitoring modes for liquid chromatography- tandem mass spectrometric methods for

determining the whole range of BPA and BPF, both being monomers of BADGE and BFDGE respectively, together with the rest of BADGE, BFDGE and their derivatives, namely BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE-$2H_2O$, and BFDGE-2HCl. The other BFDGE-related analytes, namely, BFDGE-H_2O, BFDGE-HCl and BFDGE-H_2O-HCl were not commercially available, therefore they were not analysed together in this series.

The LC-MS/MS method reported in this chapter therefore serves as an additional tool to confirm and quantitate the bisphenolic analytes by HPLC and UPLC, described in Chapters 3 and 4, respectively, which will be especially useful for analyzing complicated food matrices where interferences may provide false positives which hinders accurate quantitation. Based on the multi-reaction monitoring (MRM) capability of the LC-MS/MS instrumentation, part of the objectives in this part of the research would be to improve on the efficiency of the method by obtaining a shorter run-time for the determination of all eleven bisphenolic analytes, since the previous HPLC method described in Chapter 3 required a relatively long run-time of 95 min to separate the analytes, which is tedious and time-consuming.

5.2 Experimental

5.2.1 Materials and Reagents

Bisphenol A (minimum purity 99 %) was purchased from TCI (Tokyo, Japan); Bisphenol F, BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE, BFDGE-$2H_2O$, and BFDGE-2HCl were purchased from Fluka (Buchs, Switzerland). HPLC grade acetonitrile and methanol; analytical grade hexane and ethyl acetate were purchased from Labscan Asia (Bangkok, Thailand). Formic acid was purchased from Kanto Chemical Co. Inc (Chuo-Ku, Tokyo, Japan); ammonium formate was obtained from Riedel-De Haen (Seelze, Germany).

Stock standard solutions were individually prepared in acetonitrile, and kept in the refrigerator for not more than three months. All working standard solutions were freshly prepared prior to use. Quantitative results were obtained by comparison against external working standards and calibration curves were plotted in the range of 50 µg/L to 1500 µg/L. Mobile phases were prepared using HPLC grade acetonitrile, filtered 0.1 % formic acid solution and filtered 5 mM ammonium formate buffer solution (pH 5.0). Oasis HLB cartridges (200 mg, 6 cc) were purchased from Waters (Milford, MA, USA) for solid phase extraction.

Stock standard solutions (100 mg/L) were individually prepared in acetonitrile, and kept in the refrigerator for not more than three months. Freshly prepared intermediate stock solutions (1 mg/L) were made during each analysis run, by diluting appropriately from the respective

stock standard solution using acetonitrile. All working standard solutions were freshly prepared daily prior to use. Mobile phases were prepared using HPLC grade acetonitrile and filtered 0.1 % formic acid solution.

5.3 Apparatus

5.3.1 LC-MS/MS

LC-MS/MS analysis in multi-reaction monitoring mode (MRM) were performed using an Agilent 1100 LC system coupled to an API 4000 Q-Trap LC-MS/MS with TurboIon Spray source (Applied Biosystems, Foster City, CA, USA). The positive LCMSMS run was used for the determination of BADGE, BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE, BFDGE-$2H_2O$ and BFDGE-2HCl, and utilized the following parameters: LC injection volume: 30 μL; run length: 20 min; column temperature: 20 ^oC; column: Agilent Zorbax Eclipse XDB-C8, 4.6 mm x 150 mm, 5 μm, (Palo Alto, CA, USA); flow rate: 350 μL/ min, where 0 – 2 min: 65: 35 A: B, (v/v); 12 – 13 min: 95: 5 A: B, (v/v); 13.5 – 20 min: 65: 35, A: B, (v/v), where A = acetonitrile, and B = filtered 0.1 % formic acid solution. The mass spectrometer was equipped with an electrospray ionisation (ESI) interface operating with a source temperature: 550 °C; ion spray: 5500 V; curtain gas pressure: 10 mbar; entrance potential: 10 V (Table 5.1).

Table 5.1 LC-MS/MS parameters of the various bisphenolic analytes determined

	Retention Time/ min	Molecular weight/ D	LC-MS/MS ESI polarity	MRM mass pair	Declustering Potential / V	Collision Energy/ eV	Cell Exit Potential (CXP)/ V
BADGE	11.70	340.4	Positive	358.4 / 135.4	32	42	12
				358.4 / 161.6	32	25	12
				358.4 / 191.5	40	20	12
				358.4 / 281.5	40	26	12
BADGE-H_2O	6.03	358.4	Positive	376.4 / 161.5	35	29	8
				376.4 / 191.5	35	28	16
				376.4 / 209.7	35	21	19
BADGE-$2H_2O$	4.19	376.4	Positive	377.3 / 135.3	96	41	12
				377.3 / 209.2	96	19	12
BADGE-H_2O-HCl	5.72	394.9	Positive	395.3 / 227.1	40	20	12
				395.3 / 209.2	50	19	9
				395.3 / 135.0	36	40	13
BADGE-HCl	12.25	376.9	Positive	394.2 / 135.5	40	50	19
				394.2 / 167.2	40	33	19
				394.2 / 191.6	40	24	19
				394.2 / 227.6	40	20	19
BADGE-2HCl	10.10	413.3	Positive	415.3 / 229.1	31	18	11
				415.3 / 135.1	31	42	11
				413.4 / 135.6	30	40	10
				413.4 / 227.7	30	20	18
BFDGE	3 peaks: 9.48 10.23 10.82	312.4	Positive	330.4 / 133.4	30	24	8
				330.4 / 159.3	30	22	8
				330.4 / 163.4	30	20	8
				330.4 / 189.4	49	17	10
BFDGE-$2H_2O$	4.13	348.4	Positive	349.5 / 107.0	41	45	6
				349.5 / 133.1	41	25	10
				349.5 / 181.1	41	15	14
BFDGE-2HCl	2 peaks: 8.67 9.44	385.3	Positive	385.2 / 107.2	33	50	9
				385.2 / 181.0	33	27	13
				385.2 / 199.1	33	13	13
BPA	6.22	228.3	Negative	227.0 / 133.1	-90	-36	-9
				227.3 / 212.4	-100	-25	-7
				227.3 / 211.1	-100	-40	-7
BPF	5.37	200.2	Negative	199.2 / 105.1	-78	-30	-17
				199.2 / 77.2	-88	-30	-7

The negative LC-MS/MS run was used for the determination of BPA and BPF, which utilized the following parameters and the same analytical column: LC injection volume: 30 µL; run length: 15 min; column temperature: 20 ^{o}C; flow rate: 350 µL/ min, where 0 – 2 min: 65: 35 A: B, (v/v); 9.0 – 9.5 min: 90: 10 A: B, (v/v); 9.8 – 15.0 min: 65: 35 A: B, (v/v), where A = acetonitrile, and B = filtered 5 mM ammonium formate buffer pH 5.0. The mass spectrometer was equipped with an electrospray ionisation (ESI) interface operating with a source temperature: 550 °C; ion spray: -4500V; curtain gas pressure: 10 mbar; entrance potential: -10 V (Table 5.1). Two mass pairs for each analyte were used in the customized quantitation method, where the most abundant pair was used for quantitation, and the second most abundant mass pair was chosen for analyte confirmation.

Prior to each series of chromatographic separations, the analytical column was conditioned for at least 15 min with acetonitrile, and equilibrated with acetonitrile : 0.1 % formic acid solution (65: 35, v/v) for positive mode, or with acetonitrile : 5 mM ammonium formate buffer (pH 5.0) (65: 35, v/v) for negative mode, for a duration of at least 15 min to provide a stable baseline for subsequent chromatographic analysis.

5.3.2 HPLC

The HPLC analysis for the additional background determination of the prepared coffee drink was carried out according to an established protocol in another paper [22] on a Shimadzu HPLC system (Kyoto, Japan) equipped with a RF-10AXL fluorescence detector. Excitation

wavelength: 235 nm; emission wavelength: 317 nm; injection volume: 20 µL; run length: 95 min; column temperature: 20 ^{o}C; column: Hypurity Elite Hypersil ODS, 5 µm, C18, 250 mm x 4 mm i.d (Alltech, MA, USA); flow rate: 0.4 mL min^{-1}, where 0 – 3 min: (30: 70 A: B, v/v); 15 min: (35: 65 A: B, v/v); 22 min: (41: 59 A: B, v/v); 85 – 85.5 min: (68: 32 A: B, v/v); 86 - 95 min: (30: 70 A: B, v/v), where A = acetonitrile, and B = HPLC grade water.

5.3.3 Fourier Transform Infrared (FTIR) Spectrophotometer

The analysis of the inner can coating was made using a Fourier-Transform Infrared (FTIR) Spectrophotometer (FTIR-8400S, Shimadzu), equipped with a specular reflectance attachment, using a Si-C sampler. The obtained spectra of transmittance in the region 4000 to 500 cm^{-1} were compared to the polymer library of known epoxy resins and other polymers, in order to identify the coating type of the can surface.

5.4 Samples

The canned drink model system was prepared in-house, using coffee as the food matrix for validation studies. Coffee was prepared by dissolving 5 g of instant dried coffee in 500 mL deionised water. Following that, 20 g of sugar and 50 mL of full cream UHT milk were added. The coffee drink was then sealed using a can seamer, in cans (measuring 82 mm H x 70 mm D ; MC Packaging Pte Ltd, Singapore) coated internally with epoxy resin.

Food analysis proficiency assessment scheme (FAPAS®) test materials (series T1224) for BADGE-2HCl, BADGE-H_2O-HCl and BADGE-HCl, and (series T1226) for BADGE and BFDGE were purchased from the Central Science Laboratory (York, UK) for additional validation studies. The fortified samples for accuracy studies were prepared by pipetting a known volume of each stock standard solution into a round bottomed flask, and gently evaporating off the solvent using a stream of nitrogen gas. Five grams of the food was then weighed into the same vessel for recovery studies using the sample preparation method as described in Chapter 3 [24].

5.5 Sample Preparation

5.5.1 Determination of canned coating type

The inner can coating of the empty can was analyzed with a reflectance-FTIR, and the chemical nature of the epoxy coating polymer was identified by comparison with reference polymer spectra in the library using the library search program.

5.5.2 Determination of bisphenolic analytes in canned coffee samples

The food samples were extracted using a validated method described in an earlier paper [22] utilizing liquid-liquid extraction and solid phase extraction for sample clean-up, but requiring reconstitution of the final sample extracts in 90: 10 (v/v) acetonitrile: 0.1 % formic acid, instead of 90: 10 (v/v) acetonitrile: water prior to LC-MS/MS analysis.

5.6 Results and Discussion

5.6.1 FTIR analyses of cans

The type of coating of the cans was further subjected to FTIR analysis to confirm the identity of the coating applied (Figure 5.1), which was found to be that of epoxy resin.

A)

B)

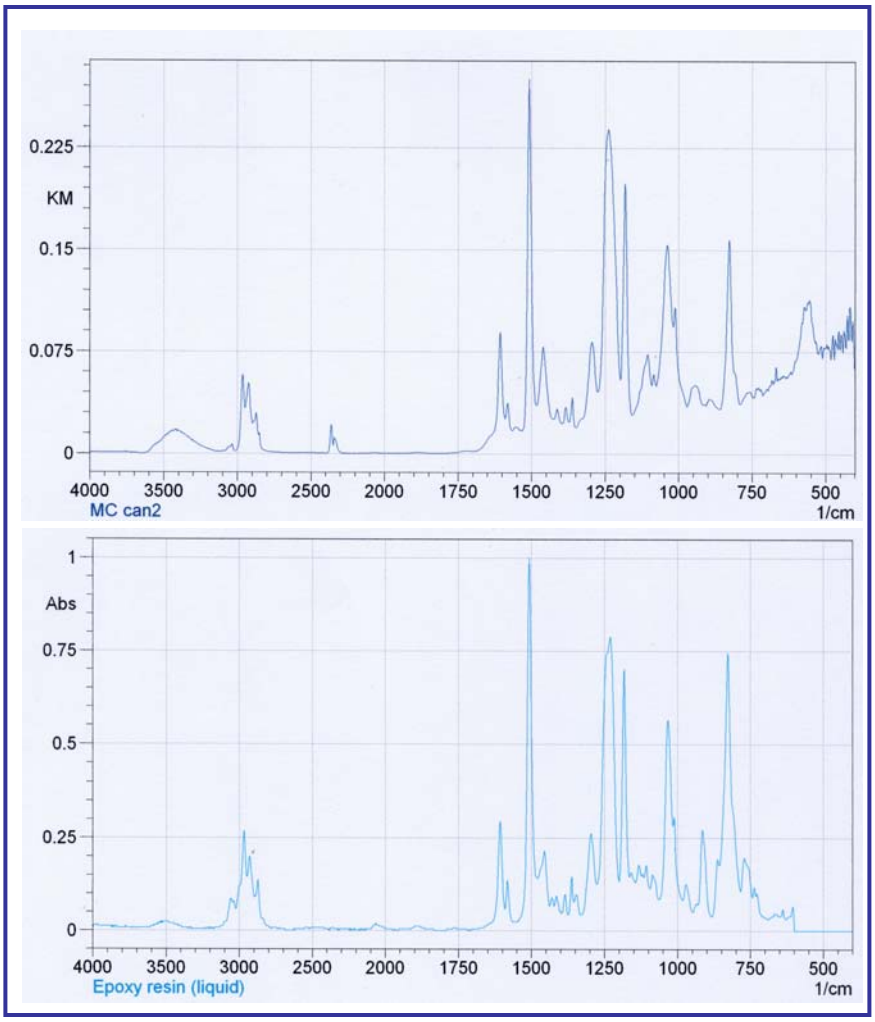

Figure 5.1 Comparison of FTIR spectrums between the internal coating applied on the test can, with respect to the FTIR spectrum of an epoxy resin – the top image (A) illustrates the FTIR scan of the internal can coating and the bottom image (B) illustrates the epoxy resin FTIR spectrum as provided by the polymer library.

5.6.2 Chromatographic Analysis

The use of both positive ESI (Figure 5.2) and negative ESI modes (Figure 5.3) for LC-MS/MS analysis was necessary to obtain optimal sensitivities for all eleven analytes in this method. During the method optimization, positive ESI modes were also tested on BPA and

BPF, but their sensitivities were far from satisfactory. The $[M-H]^-$ parent anion for both BPA and BPF was more abundant for multi-reaction monitoring studies, hence negative ESI-mode was finally chosen for their determination. The chromatographic separation required gradient elution for optimum separation of all eleven structurally-similar analytes, and the retention times of the various analytes are listed in Table 5.1. From the chromatogram, three structural isomers of BFDGE (ortho-ortho, ortho-para, and para-para) and two structural isomers of BFDGE-2HCl could also be discriminated by the chromatographic separation (Figure 5.2).

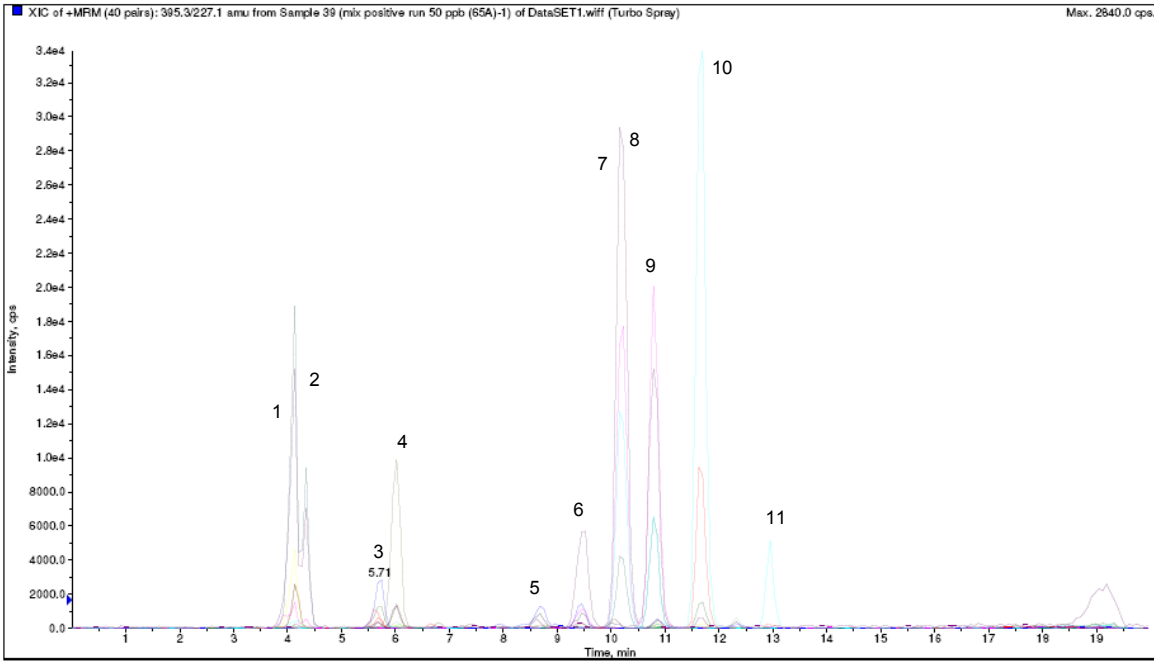

Figure 5.2 Positive ESI-LCMS/MS chromatogram illustrating nine bisphenolic analyte peaks at 50 µg/L, where 1. BFDGE-2H_2O, 2. BADGE-2H_2O, 3. BADGE-H_2O-HCl. 4. BADGE-H_2O, 5. BFDGE-2HCl, 6. BFDGE-1, 7. BFDGE-2, 8. BFDGE-2HCl, 9. BFDGE-3, 10. BADGE, 11. BADGE-HCl

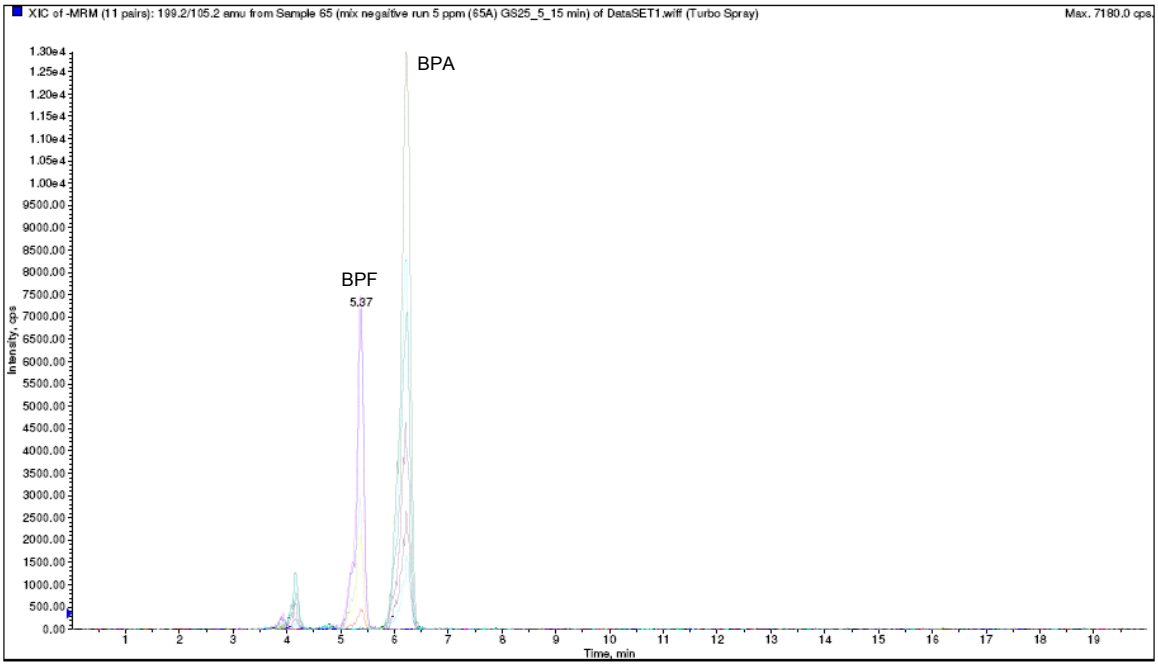

Figure 5.3 Negative ESI-LC-MS/MS chromatogram of BPA and BPF at 5 mg/L

During the method optimization, the aqueous modifier was also varied using dilute formic acid (FA; 0.1 %, 0.5 % and 1 % v/v), as well as 5 mM ammonium formate (AF; pH 2.5, 3.0, 4.0 and 5.0). For the positive ESI mode, 0.1 % formic acid was chosen as the best aqueous modifier since it had optimal sensitivities for all of the components, and it was observed that the 5 mM ammonium formate (pH 2.5, 3.0, 4.0, and 5.0) resulted in poorer sensitivities for BADGE-H_2O-HCl, BADGE-2H_2O, BADGE-2HCl, and BFDGE-2HCl. For the negative ESI mode, ammonium formate buffer (5 mM, pH 5.0) was chosen as it provided the best sensitivities, in terms of peak areas, for both BPA and BPF (Figure 5.4).

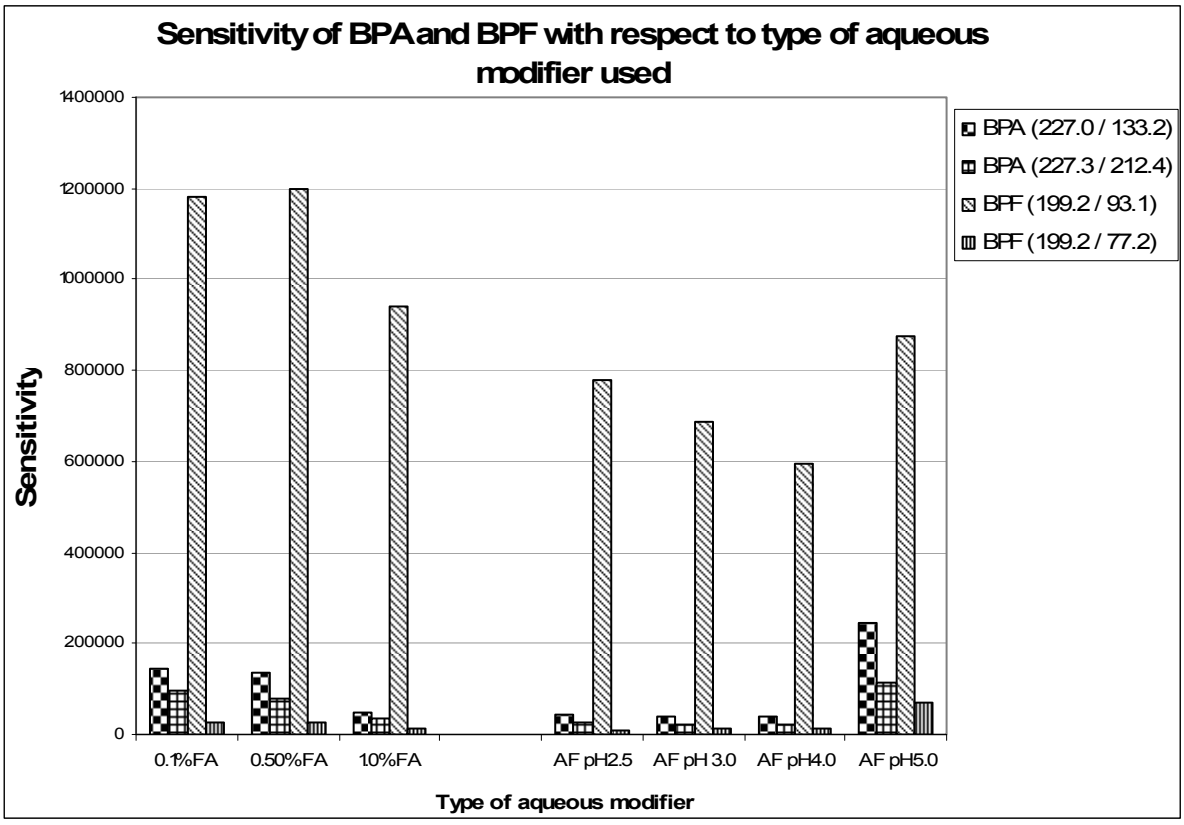

Figure 5.4 Sensitivities of BPA and BPF in negative ESI mode, with respect to the type of aqueous modifier used.

5.6.2 Method Validation

5.6.2.1 Linearity, Range, LOD and LOQ and Robustness

The eleven analytes in the positive LC-MS/MS run were evaluated for linearity using concentration levels of 50, 100, 250, 500, 750, 1000 and 1500 µg/L, and the correlation coefficients obtained were all ≥ 0.99. The limits of detection (LOD) of the analytical method, calculated from the levels of the various analytes that provided signals equivalent to three times the standard deviation of noise on analysis, were 5 µg/kg; the limits of quantitation

(LOQ), calculated from the concentration of the analytes that provided signals equal to ten times the signal to noise on analysis, were 15 µg/kg (Table 5.2). These values are conservative estimates and are varied over the course of the project.

Table 5.2 LODs, LOQs and concentration ranges of the various analytes by the reported LC-MS/MS method in this chapter, and the HPLC method (discussed in Chapter 3).

	LC-MS/MS			HPLC		
Analytes	**LOD of analyte in food (µg/kg)**	**LOQ of analyte in food (µg/kg)**	**Concentration Range/ (µg/L)**	**LOD of analyte in food (µg/kg)**	**LOQ of analyte in food (µg/kg)**	**Concentration Range/ (µg/L)**
Bisphenol A (BPA)	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
Bisphenol A diglycidyl ether (BADGE)	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
BADGE-H_2O	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
BADGE-HCl	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
BADGE-H_2O-HCl	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
BADGE-2HCl	5.00	15.0	50 - 1500	15.00	49.5	100 - 2000
BADGE-2H_2O	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
Bisphenol F (BPF)	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
Bisphenol F diglycidyl ether (BFDGE)	5.00	15.0	50 - 1500	15.00	49.5	100 - 2000
BFDGE-2HCl	5.00	15.0	50 - 1500	10.00	33.0	100 - 2000
BFDGE-2H_2O	5.00	15.0	50 - 1500	15.00	49.5	100 - 2000

5.6.2.2 Precision and Accuracy

Precision was assessed by analyzing a 50 µg/L standard solution containing all eleven analytes using the optimized method with 8 repetitions. The resulting RSD % was then

calculated by dividing the standard deviation by the mean, and multiplying the value by 100 %; results ranged from 0.67 to 4.60 % for the precision tests (n = 8).

The accuracy of the method was assessed at 40 µg/kg. Eight fortified coffee samples were extracted and analyzed using the optimized conditions. Excellent percentage recoveries ranging from 87.4 to 106 % were obtained with acceptable variation (RSD %: 3.4 to 6.1 %; Figure 5.5).

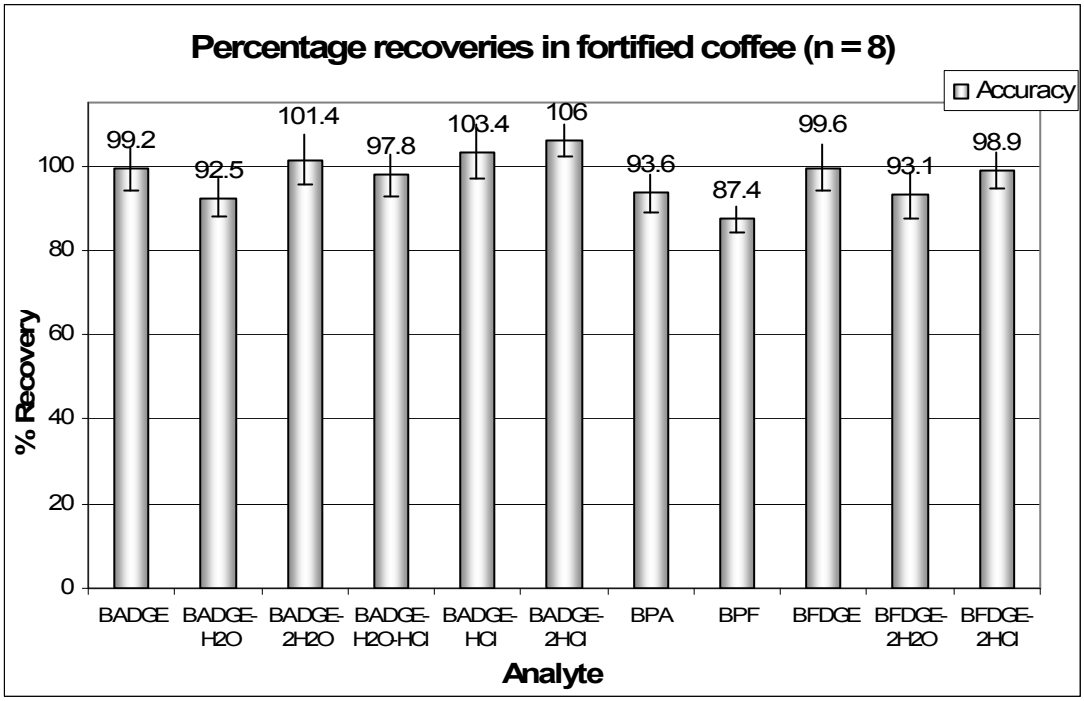

Figure 5.5 Percentage recoveries (n = 8) in fortified coffee at 40 µg/kg.

To further ensure the quality of the analytical method, two FAPAS® test materials (T1224 and T1226) were analyzed in duplicate together with the validation studies. The mean values

obtained for T1224 test material were 461, 451 and 78 µg/kg for BADGE-H_2O-HCl, BADGE-2HCl, and BADGE-HCl respectively (RSD 3.51; 1.91; 6.24 %); and 1431 and 870 µg/kg for BADGE and BFDGE, respectively (RSD 7.32 %; 9.75 %) in test material T1226 (Table 5.3). These experimentally obtained values were in good agreement with the assigned FAPAS values.

Table 5.3 Comparison of experimental results for T1224 and T1226, analysed in duplicate, with respect to the assigned values from FAPAS.

FAPAS Test Material	Analyte	Assigned Value (µg/kg)	Obtained Experimental Values (µg/kg)	Percentage Recovery (%)
T1224	BADGE-H_2O-HCl	491	461 ± 16	93.9
	BADGE-2HCl	477	451 ± 7	94.5
	BADGE-HCl	90	78 ± 5	86.7
T1226	BADGE	1483	1431 ± 36	96.5
	BFDGE	949	870 ± 85	91.7

5.6.2.3 Selectivity and Specificity

Figure 5.6 shows a HPLC chromatogram for the eleven bisphenolic analytes of the canned coffee sample that was prepared for the validation studies. Slight interferences in the background near the retention times of BFDGE-2H_2O, BADGE-2H_2O were observed, and

would have otherwise been deduced to contain small amounts of the two contaminants without a subsequent confirmatory analysis.

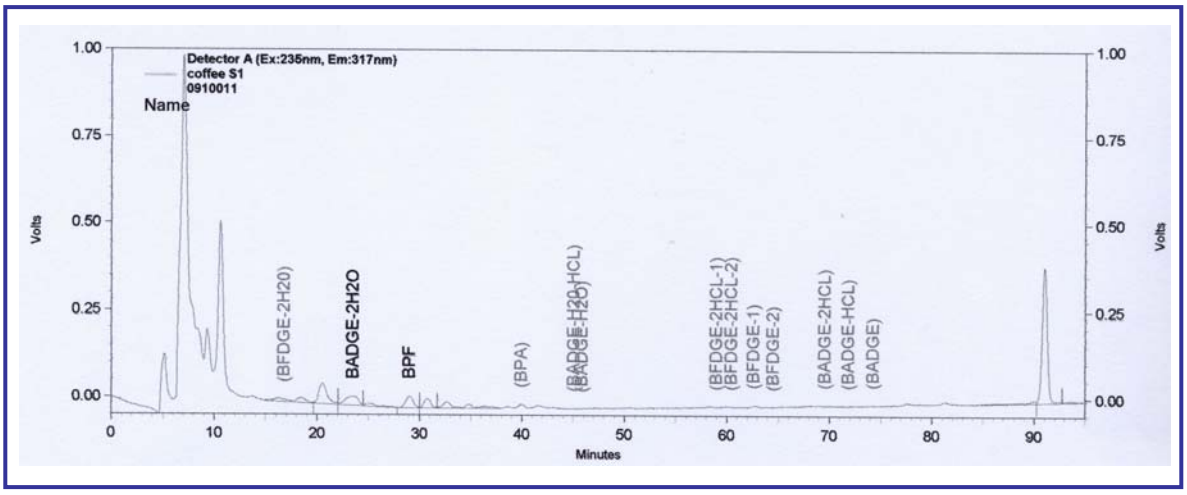

Figure 5.6 HPLC chromatogram of in-house canned coffee blank, analyzed separately on the Shimadzu system, as described in Section 5.3.2

However, when re-analyzed using the multi-reaction monitoring mode - HPLC tandem MS described in this method for confirmatory studies, the sample was found to be negative for BADGE-2H_2O, and to contain trace amounts (10 µg/kg) of BFDGE-2H_2O (Figure 5.7).

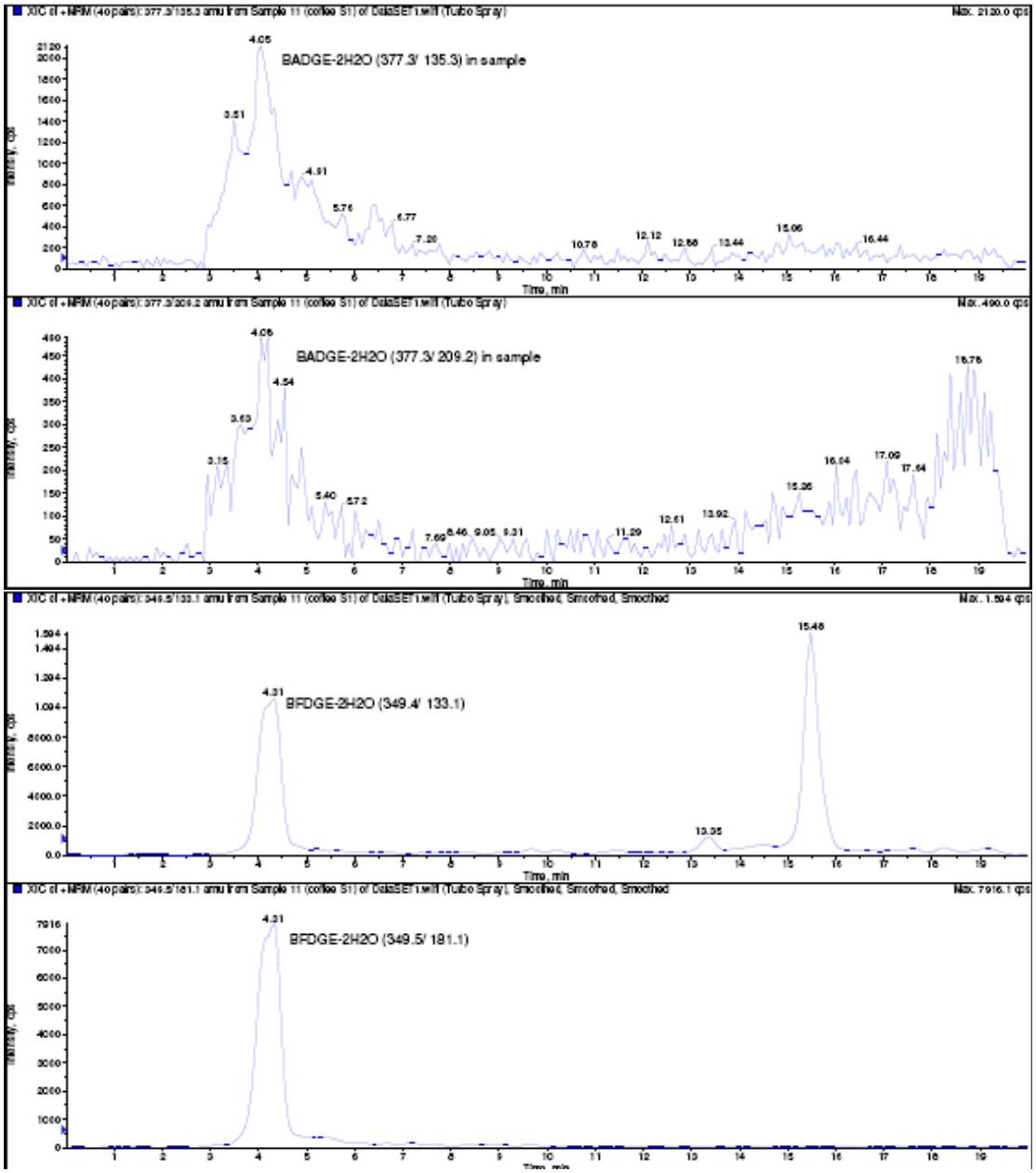

Figure 5.7 Confirmation of bisphenolic analytes (BADGE-2H_2O and BFDGE-2H_2O) from the canned coffee sample.

The additional selectivity of the method for the analytes over the matrix, and the specificity of the MRM-mass pairs have important consequences for food regulatory agencies for the

enforcement of the food laws. In addition, the LC-MS/MS method described also benefits the laboratory considerably in terms of the shorter run-time of 20 min (with respect to the HPLC method), and improves the laboratory throughput appreciably.

5.7 Conclusions

The developed method for the simultaneous determination of the eleven bisphenolic analytes has been shown to be a more selective and confirmatory method for determination of the various bisphenolic analytes at low limits of detection. The specificity of the method towards the bisphenolic analytes from the model coffee drink sample, which consists of a more complicated matrix as compared to most other drink-types, has been demonstrated. The excellent validation data obtained, and the satisfactory results performed on the two FAPAS materials indicate that the method is fit for its intended use. In addition, the lower LODs and LOQs determined by this method enhances the sensitivity of this method on complicated food matrices, which will be important for routine laboratories; the relatively short analysis time of 20 min, as compared to the 95 min run-time of the HPLC method (Chapter 3) has reduced the analysis time by more than four times, and this allows for the provision of throughput for the laboratory.

5.8 References

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CHAPTER 6

Chapter 6

Measurement Uncertainty of Bisphenol A, Bisphenol F, Bisphenol A Diglycidyl Ether and its derivatives, and Bisphenol F Diglycidyl Ether and its derivatives by Reversed Phase- High Performance Liquid Chromatography with Fluorescence Detection

6.1 Introduction

Method validation is an important requirement in the practice of analytical chemistry. It is the process of defining an analytical requirement, and confirming that the method under consideration has performance characteristics and capabilities consistent with what the application requires. In other words, it determines the fitness of purpose of the intended analysis. Methods need to be validated in the following circumstances: when an established method has been revised to incorporate improvements or extended to a new problem; when a new method has been developed for a particular problem; when quality control indicates that the established method has been changing with time; or when an established method is to be used in a different laboratory, with different analysts, or with different instrumentation(s) [1].

All analytical measurements have measurement errors, which can be attributed to gross errors (blunders), systematic errors, or random errors. Hence, it is important to evaluate possible errors in the estimated analytical value, as provided by the test results, a process also known

as the uncertainty measurement. This ensures that the analytical data is technically sound and defensible, especially when there are cases of disputes between inter-laboratories, and also allow for the improvement of confidence associated with the reliability of analytical results of food samples, that can allow for the removal of barriers to international trade.

The uncertainty is usually not due primarily to the errors by the analysts. Rather, it is inherent in the measuring process. The key principles of method validation and uncertainty estimation requires that the validation studies to be representative of the normal operation of the method. In addition, the studies must encompass the complete method which includes the representative range of sample matrices, as well as the representative linear range of the analyte(s). Therefore, an accurate estimation of the measurement uncertainty would require the combination of the estimations of precision, trueness (bias), as well as other uncertainty contributions such as the purity of certified reference standards, data from recognized proficiency programmes, as well as any sub-sampling errors, that could possibly contribute to the accuracy of the analytical result.

Measurement Uncertainty = precision + bias + others **(Equation 6.1)**

In this work, we estimate the measurement uncertainty of bisphenol A (BPA) and bisphenol F (BPF), together with the wide range of bisphenol A diglycidyl ether (BADGE), bisphenol F diglycidyl ethier (BFDGE) and their derivatives (BADGE-H_2O, BADGE-$2H_2O$, BADGE-H_2O-HCl, BADGE-HCl, BADGE-2HCl, BFDGE-$2H_2O$, and BFDGE-2HCl based on the precision data, bias, as well as the uncertainties associated with the purities of the bisphenolic

analytes, since these factors are more relevant in this context with respect to the rest of the parameters described earlier. The following paragraphs therefore illustrates the process of estimating the precision, accuracy, as well as other sources of uncertainties of the method to compute and determine the measurement uncertainty of the method for the determination of bisphenolic analytes in canned food, using solid-phase extraction clean-up and RP-HPLC, as described in greater detail in Chapter 3.

6.2 Precision study (Inter-day)

Precision refers to the closeness of the agreement between the results of successive measurement of the same analyte carried out under the same conditions of measurement. The inter-day precision of the bisphenolic analytes was estimated through the repeated analysis (n = 8) of the same standard at 100 μg/L over 3 days, using the same instrument, with the objective of determining the standard uncertainty due to run- to- run variation of the overall analytical process over 3 days (Table 6.1).

Table 6.1 Interday precision results (n =8) determined using a 100 µg/L mixed standard solution containing all bisphenolic analytes performed over three days, with RSD % values in parenthesis.

Interday Precision Results (n = 8) Mean Concentration / (µg/L)						
Analytes	**Day 1**	**Day 2**	**Day 3**	**Mean Concentration (Day 1) / (µg/L)**	**Standard Deviation (Day 1)**	**RSD % (Day 1)**
BADGE-2H_2O	99.39 (3.13)	98.89 (1.50)	99.62 (3.28)	99.39	3.11	3.13
BPA	98.63 (1.54)	97.70 (1.88)	98.43 (2.48)	98.63	1.52	1.54
BADGE-H_2O-HCl	99.39 (1.92)	98.05 (1.76)	95.89 (3.46)	99.39	1.91	1.92
BADGE-H_2O	99.24 (1.56)	98.84 (1.62)	98.79 (1.35)	99.24	1.55	1.56
BADGE-2HCl	101.69 (2.79)	101.20 (3.70)	98.46 (3.54)	101.69	2.84	2.79
BADGE-HCl	96.83 (1.55)	96.944 (1.62)	97.49 (3.07)	96.83	1.50	1.55
BADGE	99.63 (0.75)	100.25 (1.03)	97.45 (3.24)	99.63	0.75	0.75
BFDGE-2H_2O	98.36 (2.78)	97.09 (4.45)	96.32 (6.95)	98.36	2.71	2.78
BPF	99.26 (1.49)	97.28 (2.10)	98.94 (1.11)	99.26	1.48	1.49
BFDGE-2HCl	98.51 (3.25)	98.13 (0.57)	98.04 (2.78)	98.51	3.20	3.25
BFDGE	93.82 (4.38)	95.08 (2.40)	96.27 (2.33)	**93.82**	**4.11**	**4.38**

In order to estimate the worst case uncertainty with respect to precision for this project, we use the largest coefficient of variation for the computation of the measurement uncertainty of the overall process (Table 6.1), using values from the first day to simplify the calculations. The uncertainty of precision studies was therefore found to be 0.0438 (BFDGE).

6.3 Bias Study

Bias refers to the difference between the expectations of the test results with an accepted reference value. The bias of the analytical procedure was investigated during the in-house validation study using spiked canned food samples. Table 6.2 provides the results of an extensive study of the recoveries from spiked canned food samples at three concentration levels: 100, 500, and 2000 µg/kg, performed in 10 replicates.

Table 6.2 Results of recovery (bias) study at 100, 500, and 2000 µg/kg levels, with RSD % values in parenthesis.

	Accuracy Results (n = 10) [RSD % in brackets]			**(100 µg/kg level)**		
	100 µg/kg level	**500 µg/kg level**	**2000 µg/kg level**			
Analytes	**Mean Recovery / %**	**Mean Recovery / %**	**Mean Recovery / %**	**Mean Recovery**	**Standard Deviation**	**RSD %**
BADGE-$2H_2O$	98.48 (2.74)	91.36 (5.39)	92.21 (3.14)	98.48	2.70	2.74
BPA	97.93 (6.50)	97.28 (3.45)	99.75 (2.27)	97.93	6.37	6.50
BADGE-H_2O-HCl	98.39 (3.63)	89.97 (2.81)	96.20 (1.07)	98.39	3.57	3.63
BADGE-H_2O	93.13 (1.93)	87.48 (8.75)	96.08 (1.12)	93.13	1.80	1.93
BADGE-2HCl	93.46 (2.27)	94.25 (8.47)	91.78 (1.13)	93.46	2.12	2.27
BADGE-HCl	97.05 (4.36)	90.11 (4.01)	91.96 (1.19)	97.05	4.23	4.36
BADGE	97.06 (2.34)	93.59 (5.31)	98.27 (0.91)	97.06	2.27	2.34
BFDGE-$2H_2O$	99.25 (3.35)	89.41 (2.38)	96.98 (3.38)	99.25	3.32	3.35
BPF	97.58 (3.42)	95.49 (3.37)	100.84 (1.29)	97.58	3.34	3.42
BFDGE-2HCl	92.72 (2.20)	94.83 (3.24)	99.59 (1.29)	92.72	2.04	2.20
BFDGE	100.49 (5.33)	97.21 (3.09)	99.97 (2.49)	100.49	5.36	5.33

Standard uncertainty U(x) of BADGE-2H2O = $\frac{sd}{\sqrt{n}} = \frac{0.027}{\sqrt{10}} = 0.0085$

Standard uncertainty U(x) of BPA = $\frac{sd}{\sqrt{n}} = \frac{0.0637}{\sqrt{10}} = 0.0201$

Standard uncertainty U(x) of BADGE-H_2O-HCl = $\frac{sd}{\sqrt{n}} = \frac{0.0357}{\sqrt{10}} = 0.0113$

Standard uncertainty U(x) of BADGE-H_2O = $\frac{sd}{\sqrt{n}} = \frac{0.0180}{\sqrt{10}} = 0.0057$

Standard uncertainty U(x) of BADGE-2HCl = $\frac{sd}{\sqrt{n}} = \frac{0.0212}{\sqrt{10}} = 0.0067$

Standard uncertainty U(x) of BADGE-HCl = $\frac{sd}{\sqrt{n}} = \frac{0.0423}{\sqrt{10}} = 0.0134$

Standard uncertainty U(x) of BADGE = $\frac{sd}{\sqrt{n}} = \frac{0.0227}{\sqrt{10}} = 0.0072$

Standard uncertainty U(x) of BFDGE-2H_2O = $\frac{sd}{\sqrt{n}} = \frac{0.0332}{\sqrt{10}} = 0.0105$

Standard uncertainty U(x) of BPF = $\frac{sd}{\sqrt{n}} = \frac{0.0334}{\sqrt{10}} = 0.0106$

Standard uncertainty U(x) of BFDGE-2HCl = $\frac{sd}{\sqrt{n}} = \frac{0.0204}{\sqrt{10}} = 0.0065$

Standard uncertainty U(x) of BFDGE = $\frac{sd}{\sqrt{n}} = \frac{0.0536}{\sqrt{10}} = 0.0169$

6.3.1 Calculation of bias based on recovery data

In a perfect situation, the recovery (R) would be exactly unity (1) but in reality, circumstances such as imperfect sample extractions, standard calibration and purity may result in

observations that differ from the ideal. We can determine the recovery for any significant departure from unity by using the Students' *t*-test, by considering the question:

"Is $|R - 1|$ greater than u_R, the uncertainty in the determination of R ?"

The significance testing can therefore be conducted as follows:

$H_0 : | R - 1| / u_R < t_c$ *R does not differ significantly from 1*

$H_1 : | R - 1| / u_R > t_c$ *R differs significantly from 1*

where *t* is the critical value [2].

To calculate the bias based on the available recovery data from the experiments, the student's t - distribution significance test was subsequently taken to test if the recoveries were significantly different from 1. For 9 degrees of freedom (n = 10), $t_c = 2.262$ according to the two-tailed critical t_α values of Students' t variables tabulated in Table 6.3. For calculation purposes, the recoveries of the various analytes at 100 µg/kg were used.

The t-test values of the recoveries (100 µg/kg level) were tabulated in Table 6.4 according to the equation 6.2:

$$t = \frac{|1 - \text{Recovery}|}{U(x)} \qquad \text{(Equation 6.2)}$$

Table 6.3 Two-tailed critical t_α values of Students' t variables at 95 % Confidence intervals.

Degrees of freedom	Critical t_α value at 95 % confidence interval
1	12.706
2	4.303
3	3.182
4	2.776
5	2.571
6	2.447
7	2.365
8	2.306
9	**2.262**
10	2.228

Table 6.4 Two-tailed Students' t values at 95 % Confidence intervals.

Analyte	Students' t-test values at 95 % confidence interval at 100 µg/kg level
BADGE-2H_2O	1.788
BPA	1.030
BADGE-H_2O-HCl	1.425
BADGE-H_2O	**12.053**
BADGE-2HCl	**9.761**
BADGE-HCl	2.201
BADGE	**4.083**
BFDGE-2H_2O	0.714
BPF	**2.283**
BFDGE-2HCl	**11.200**
BFDGE	0.237

From the above calculations, the values of $t_{BADGE\text{-}H2O}$, $t_{BADGE\text{-}2HCl}$, t_{BPF} and $t_{BFDGE\text{-}2HCl}$, are greater than the corresponding critical value, t_c = 2.262, which indicate that the recoveries would be significantly different from 1, but in the normal application of the method, no corrections were applied to the analytical results as the recoveries at 100 µg/kg, 500 µg/kg, and 2000 µg/kg were usually well within 85 – 115 %. The uncertainty must be increased to take account of the fact that the recovery had not been corrected for. To estimate the worst case scenario for the estimation of the measurement uncertainty, we use the largest increased uncertainty, U_i (x) of BADGE-H_2O at 100 µg/kg level :

$$U_{i(100)} = \sqrt{\left(\frac{1-\text{Recovery}}{k}\right)^2 + (U(x))^2} = \sqrt{\left(\frac{1-0.9313}{2}\right)^2 + (0.0057)^2} = 0.0348$$

(Equation 6.3)

Therefore, the uncertainty for bias was calculated as 0.0348.

6.4 Other Sources of Uncertainty

6.4.1 Balances/ Volumetric Measuring Devices

All balances and the important measuring devices were under regular control.

Precision and recovery studies have already taken into account the influence of the calibrations of the different volumetric measuring devices, such as volumetric flasks and pipettes, which were calibrated prior to use.

6.4.2 Sample Homogeneity

The food samples were shaken for at least 20 min prior to sample preparation on the rotating platform, and were assumed to be homogeneous.

6.5 Reference material purity

The uncertainties associated with the standards were calculated based on the standard uncertainties of their purities, as given in the certificates of analysis (Table 6.5).

Table 6.5 Purities and uncertainties associated with the standards, as given in the certificates of analysis.

Name	Purity / %	Uncertainty associated with purity
BFDGE-2H2O	96.6 (98.3 ±1.7)	0.0098
BADGE-2H2O	98.1 (99.05 ±0.95)	0.005
BPF	98 (99 ± 1)	0.0057
BPA	99.1 (99.55 ± 0.45)	0.003
BADGE-H2O-HCL	96.9 (98.45 ± 1.55)	0.0089
BADGE-H2O	95.8 (97.9 ± 2.1)	0.012
BFDGE-2HCL	92.4 (96.2± 3.8)	0.022
BFDGE	96.5 (98.25± 1.75)	0.010
BADGE-2HCL	99.7 (99.85±0.15)	0.0009
BADGE-HCL	93.2 (96.6 ± 3.4)	0.0196
BADGE	97 (98.5 ± 1.5)	0.0086

6.6 Summary of Uncertainty Estimation of BADGE method

Table 6.6 Overall uncertainty estimation of BADGE procedure

	Canned Food				
	Description	**Value x**	**Standard uncertainty u(x)**	**Relative Standard uncertainty u(x)/x**	**Comments**
A	**Precision**	1	-	-	Evaluation of precision of 100 µg/L standard solutions (n=8)
	BADGE-2H_2O		0.0311	0.0311	
	BPA		0.0152	0.0152	
	BADGE-H_2O-HCl		0.0191	0.0191	
	BADGE-H_2O		0.0155	0.0155	
	BADGE-2HCl		0.0284	0.0284	
	BADGE-HCl		0.0150	0.0150	
	BADGE		0.0075	0.0075	
	BFDGE-2H_2O		0.0271	0.0271	
	BPF		0.0148	0.0148	
	BFDGE-2HCl		0.0320	0.0320	
	BFDGE		**0.0411**	**0.0411**	
B	**Bias**	0.9313	0.0348	0.0374	Evaluation of bias using increased uncertainty of BADGE-H_2O
C_1	**Purity**	-	-	-	As per certificate of analysis
	BADGE-2H_2O	0.966	0.0098	0.010	
	BPA	0.981	0.005	0.005	
	BADGE-H_2O-HCl	0.98	0.0057	0.006	
	BADGE-H_2O	0.991	0.003	0.003	
	BADGE-2HCl	0.969	0.0089	0.009	
	BADGE-HCl	0.958	0.012	0.013	
	BADGE	0.924	0.022	0.024	
	BFDGE-2H_2O	0.965	0.01	0.010	
	BPF	0.997	0.0009	0.0009	
	BFDGE-2HCl	0.932	0.0196	0.021	
	BFDGE	0.97	0.0086	0.009	
	$U(P_{op})/ P_{op}$	--	--	0.0686	

Combined uncertainty = $\sqrt{(A)^2 + (B)^2 + (C)^2}$

=

$$\sqrt{(0.0411)^2 + (0.0374)^2 + (0.010)^2 + (0.005)^2 + (0.006)^2 + (0.003)^2 + (0.009)^2 + (0.013)^2 + (0.024)^2 + (0.010)^2 + (0.0009)^2 + (0.021)^2 + (0.009)^2}$$

= **0.0686**

Table 6.6 illustrates the summary of the various estimations of uncertainty components into the estimation of measurement uncertainty associated with the overall BADGE procedure. The expanded uncertainty U (P_{op}) of the overall procedure at 95% confidence level is obtained by multiplying the combined standard uncertainty with a coverage factor (k) of 2, giving 0.14, or about 14 %. The value obtained is a rough estimate of the combined measurement uncertainty. The accurate measurement uncertainty budgeting of the result, as stated in [3], is beyond the scope of this thesis.

6.7 Conclusions

The measurement uncertainty of the BPA, BPF, BADGE and its derivatives, as well as BFDGE and its derivatives in canned food by solid phase extraction clean-up and liquid chromatography with HPLC using fluorescence detection had been estimated, and the expanded uncertainty estimate was only about 14 % of the analytical result, based on the worst case scenarios.

The relatively small uncertainty measurement reflected well on the validated method, and provided reliability on the use of this proposed method for analyzing the bisphenolic content in the wide range of canned foods.

6.8 References

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CHAPTER 7

Chapter 7

Determination of Isopropyl-9H-thioxanthen-9-one in Packaged Beverages by Solid Phase Extraction Clean-up and Liquid Chromatography with Tandem Mass Spectrometry Detection

7.1 Introduction

As a photo-initiator in UV cured inks, isopropyl-9H-thioxanthen-9-one (ITX) triggers the radical polymerization of the acrylic component of such inks to allow the liquid ink film to dry. Currently, inks applied to food packaging materials are not covered by specific European legislation, however, materials and articles intended to come into contact with foods should comply with the general criteria laid down in Article 3 of Regulation (EC) No. 1935/2004, stating that materials and articles in contact with food shall be manufactured in such a way that they do not transfer their constituents to food in quantities which could change the composition of the food or bring about unacceptable deterioration in the organoleptic characteristics thereof [1]. As the local regulatory agencies adhere to the European legislation, this investigation on printed packaged beverages was therefore initiated in response to the recent food survey data published.

ITX has recently been found in foods such as ready-to-feed infant formula, resulting in the withdrawal of more than 30 million litres of milk by producers in France, Italy, Spain and

Portugal in November 2005. According to the European Food Safety Authority (EFSA), although ITX has not shown any indication for harmful effects on human health, its presence in foods is still considered undesirable. Therefore, based on available toxicological data, the Federal Institute for Risk Assessment (BfR) has deduced a specific migration limit of 0.05 mg/kg.

Presently, there are very few methods available in the current literature for the analysis of ITX in foods as well as in their packaging materials. Morlock *et al.* has developed a novel methodology for the extraction of ITX in milk, yoghurt and fat using accelerated solvent extraction with high performance thin-layer chromatography coupled with mass spectrometry for detection of ITX down to levels of 1 μg/kg in fatty food [2]. It was shown that in the HPTLC sample preparation sample preparation can be kept simple and rapid, however, the mean recovery rates of ITX could be further improved. Other methods available in the literature included the use of GC-MS for the determination of ITX in aqueous simulants [3,4].

The method provides a simple extraction protocol for determining very low levels of ITX from beverages packaged in printed food packaging materials with excellent recovery data. This is a significant improvement from the existing methodologies in terms of sensitivity, accuracy and method turn-around-time. It comprises the addition of an internal standard, deuterium-labelled 2-isopropyl-[$^{2}H_{7}$]-thioxanthen-9-one (ITX-d_{7}; molecular weight 261 D) prior to extraction and clean-up using solid-phase extraction. A range of beverages and their food packaging materials were also analysed to provide background information on the extent of ITX contamination present in the samples purchased locally in Singapore [5].

Subsequently, there were also other published methods in the literature for 2-ITX by GC/MS and LC/MS in packaged food beverages [6], ITX by LC-MS [7], 2-ITX and 4-ITX by LC tandem MS [8], and 2-ITX by HPLC-DAD/FLD [9].

7.2 Experimental

7.2.1 Materials and Reagents

ITX ($C_{16}H_{14}OS$, molecular weight 254 D; 97% mixture of 2- and 4- isomers) was purchased from Sigma-Aldrich Corporation (St Louis, MO, USA); ITX-d_7 (Figure 7.1) was purchased from WITEGA Laboratorien (Berlin, Germany). Carrez reagent I (potassium hexacyanoferrate (II) trihydrate) (Sigma-Aldrich; St Louis, MO, USA) and carrez reagent II (zinc acetate) (Perkin-Elmer; Waltham MA, USA) were purchased separately. HPLC grade acetonitrile, and methanol were purchased from Labscan Asia (Bangkok, Thailand). Oasis HLB cartridges (60 mg, 3 mL) were purchased from Waters (Milford, MA, USA) to perform solid phase extraction of the samples.

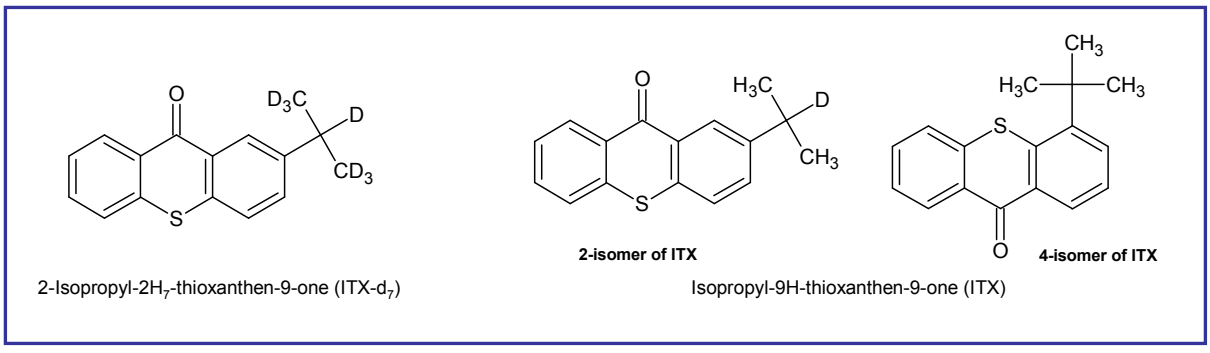

Figure 7.1 Chemical structures of ITX-d_7 and ITX (2- and 4- isomers).

Stock standard solutions (100 mg/L) were individually prepared in acetonitrile, and kept in the refrigerator at 5 °C for not more than three months. Freshly prepared intermediate stock solutions (1 mg/L) were made during each analysis run, by diluting appropriately from the respective stock standard solution using acetonitrile. All working standard solutions were freshly prepared daily prior to use. Mobile phases were prepared using HPLC grade acetonitrile and filtered 0.1 % formic acid solution.

7.3 Apparatus

LC-MS/MS analyses were performed using an Agilent 1100 LC system coupled to an API 4000 Q-Trap LC-MS/MS with TurboIon Spray source (Applied Biosystems, Foster City, CA, USA). LC injection volume: 20 µL; run length: 10 min; column temperature: 20 ^{o}C; column: Thermo BDS Hypersil C18, 5 µm, 100 mm x 3 mm I.D. (Thermo Electron, Waltham, MA, USA); flow rate: 350 µL/ min, where 0 – 1 min: 80: 20 A: B (v/v); 5 – 6 min: 95: 5 A: B (v/v); 6.1 – 10 min: 80: 20 A: B (v/v), where A = methanol, and B = filtered 0.1 % formic acid solution. Prior to each series of chromatographic separations, the analytical column was conditioned for 15 min with methanol, and equilibrated with methanol : 0.1% formic acid solution (80: 20, v/v) for at least 10 min to provide a stable baseline for subsequent chromatographic analysis. The mass spectrometer (MS) was carried out using the multiple reaction monitoring (MRM) mode, and was equipped with an electrospray ionisation (ESI)

interface operating in the positive mode. MS source temperature: 450 °C; ion spray: 5500V; curtain gas pressure: 10 mbar; declustering potential: 84 V; entrance potential: 10 V.

Method robustness was determined using 2 different analytical columns: Thermo BDS Hypersil C18, 5 μm, 100 mm x 3 mm I.D. (Thermo Electron; Waltham MA, USA), and Inertsil ODS-3, 3 μm, 100 mm x 3 mm I.D. (G. L. Sciences; Tokyo, Japan).

7.4 Samples

Thirty-nine types of beverages consisting of milk, juice, tea and yoghurt drink samples were obtained locally. These samples were chosen as their primary food packaging material had moderate to heavy printing on the surface. For each analytical run, a sample blank as well as a fortified milk and/or juice sample was prepared to estimate the degree of recovery. The fortified sample was prepared by gently evaporating a small volume of ITX and ITX-d_7 intermediate-stock standard solution that was pipetted accurately into a round bottom flask with a micropipette, using nitrogen gas. This fortified sample was later subjected to the described sample preparation method below. In order to ensure accurate analytical results, all reagents used in the analysis were prepared fresh and analyzed separately to ensure that they were free from any interfering contaminants.

7.5 Sample Preparation

Food - After 500 µL of 1 mg/L internal standard solution had been spiked into a flat round bottomed flask, the solvent was evaporated to dryness using nitrogen gas. Ten grams of sample was weighed into the vessel, and 100 mL of acetonitrile : water containing 1 % (v/v) of Carrez reagent 1 and 1 % (v/v) of Carrez reagent 2 (60: 40, v/v), measured in a 100 mL standard flask, was transferred to the sample. The resulting mixture was shaken for 20 min, and transferred to a centrifuge tube to be centrifuged at 4000 rpm for 15 min. Ten millilitres of the supernatant solution was pipetted out, and diluted to 30 mL with deionised water. 6 mL of the diluted sample was loaded onto the SPE cartridge that was previously conditioned with 3 mL of methanol and equilibrated with 3 mL of water. After washing the cartridge with 3 mL of water and 3 mL of acetonitrile: water (20: 80, v/v), the analytes were eluted with 4 mL of acetonitrile. The resulting eluate was blown dry with nitrogen, reconstituted with 1 mL of acetonitrile: 0.1 % formic acid (95: 5, v/v) acetonitrile: water, and filtered through 0.45 µm nylon filters into HPLC vials prior to LC-MS/MS analysis.

Food packaging material - 200 mL of acetonitrile was added to a glass bottle containing cut pieces (of approximately 2 cm by cm) of the food packaging material that were in contact with the food to extract the residual photoinitiators. The contents were soaked in the capped glass bottle for 24 h. One mililitre of the aliquot was pipetted into a HPLC vial after shaking the total contents in the glass bottle for 5 min. The extract was blown to dryness using nitrogen gas, and reconstituted with 990 μL of acetonitrile : 0.1 % formic acid (95:5, v/v), and 10 μL of internal standard.

7.6 Results and Discussion

7.6.1 Optimization of MS parameters

From an analysis of the product ions of ITX (Figure 7.2), three intense collision products at m/z 213.3, 184.1, and 152.2 were observed, of which the mass pairs (255/ 213.3) and 255/ 184.1) were subsequently chosen for monitoring the presence of ITX using the MRM mode. The individual parameters for each mass pair are given in Table 7.1.

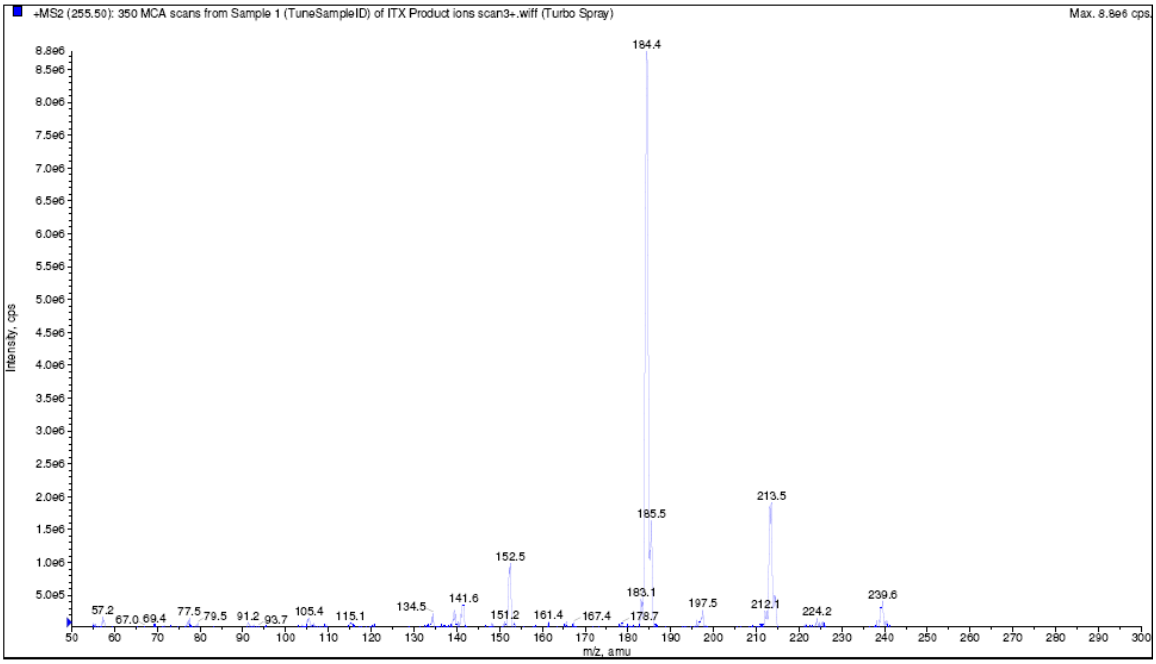

Figure 7.2 Product ion scan mass spectra of 500 µg/L ITX standard in positive mode.

Table 7.1 MS parameters for ITX and ITX-d_7 mass pairs.

Analyte	Molecular weight / u	Parent ion (Q1) / u	Monitored mass pairs	Collision Energy/ V	Cell Exit Potential/ V
ITX	254	255	255 / 213.3	30	11
			255 / 184.1	57	8
ITX- d_7	261	262.4	262.4 / 214.5	30	10
			262.4 / 185.4	56	9

7.6.2 Method Validation

7.6.2.1 Linearity, Range, LOD and LOQ

The linearity of ITX was evaluated by inspecting the detection signals as a function of analyte concentration, with the aid of a regression line by the method of least-squares, using concentration levels of 0.10, 0.50, 1.00, 10.0, 50.0, and 100.0 µg/L (Figures 7.3 and 7.4). The correlation coefficients obtained were all ≥ 0.999. The linearity of the analyte in the given concentration range, as well as their respective method detection limit (MDL) and the method quantitation limit (MQL) are provided in Table 7.2.

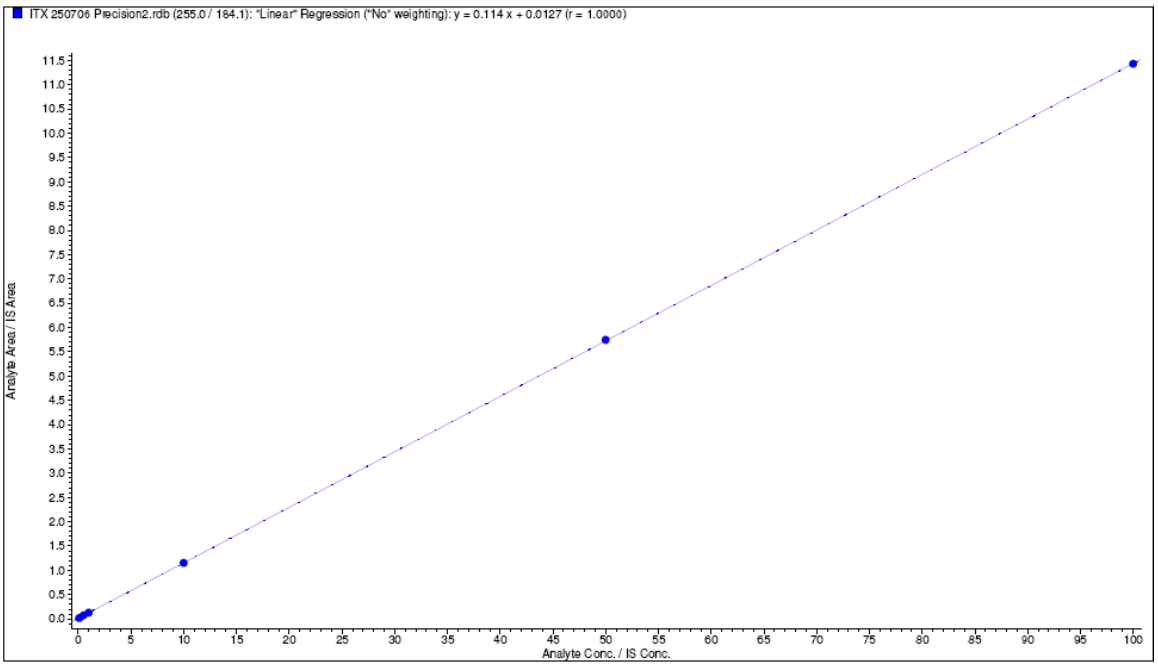

Figure 7.3 Linearity of ITX mass pair (255.0 / 184.1) in the range of 0.1 – 100.0 µg/L.

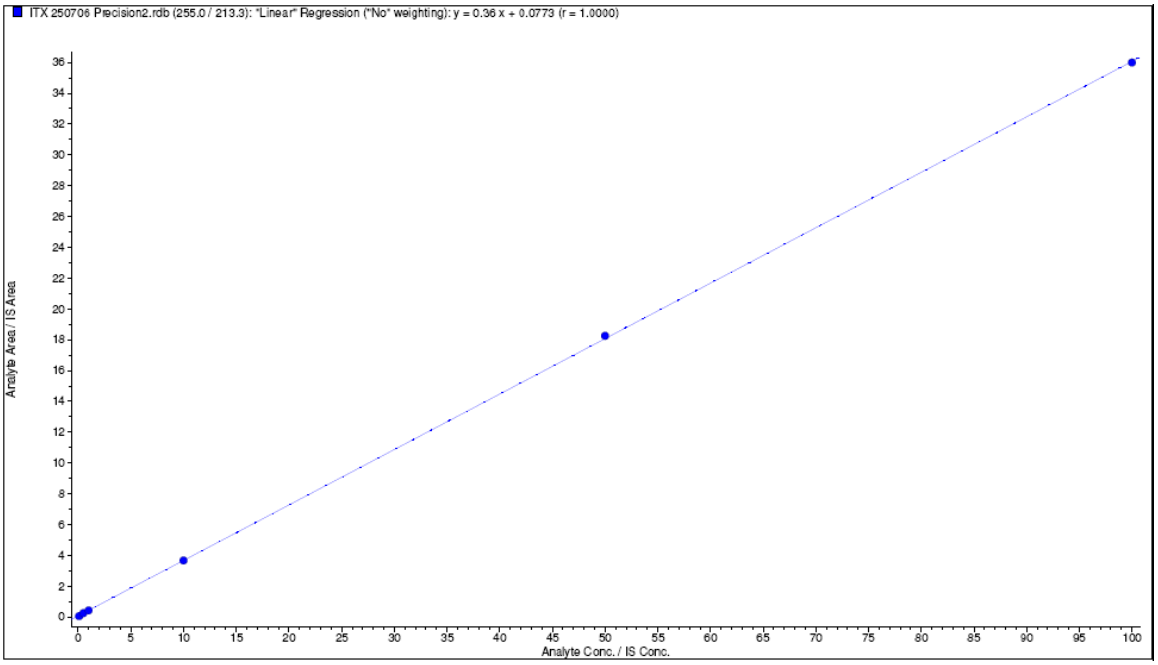

Figure 7.4 Linearity of ITX mass pair (255.0 / 213.3) in the range of 0.1– 100.0 µg/L.

Table 7.2 MDL, MQL values of ITX (with reference to the internal standard, 262.4 / 214.5) analysed within the range of 0.1 µg/L to 100 µg/L. Precision data (both interday (n=10), and intraday (n=5)); and mean recoveries of ITX are provided with the RSD values stated within parenthesis.

MDL / (µg/kg)	MQL / (µg/kg)	Intraday Precision (n = 10) RSD	3-Day Interday Precision (n = 10) RSD	Mean recovery (%) at 2.5 (µg/kg) level (n= 8)	Mean recovery (%) at 100 (µg/kg) level (n=8)	Mean recovery (%) at 500 (µg/kg) level (n=8)
0.15	0.50	0.97%	0.72%	99.95 (2.56)	101.02 (1.58)	98.98 (1.00)

7.6.2.2 Precision and Accuracy

Precision was assessed on a 0.1 µg/L standard solution (Figure 7.5) for 3 consecutive days, with 10 replicates performed each day (Table 7.2). The RSD was then calculated by dividing

the standard deviation by the mean, and the value multiplied by 100 %. The RSD for intraday precision (n = 5) ranged from 0.27 – 0.72 %; the RSD for interday precision (n = 10) ranged from 0.52 – 0.76 %. The accuracy of the method was assessed at three concentration levels: 2.50, 100 and 500 µg/kg. Eight fortified milk samples at each concentration level were extracted and analyzed under the optimized conditions. Extremely good percentage recoveries were obtained (97.0 - 103.0 %) with excellent RSD (1.00 - 2.56 %).

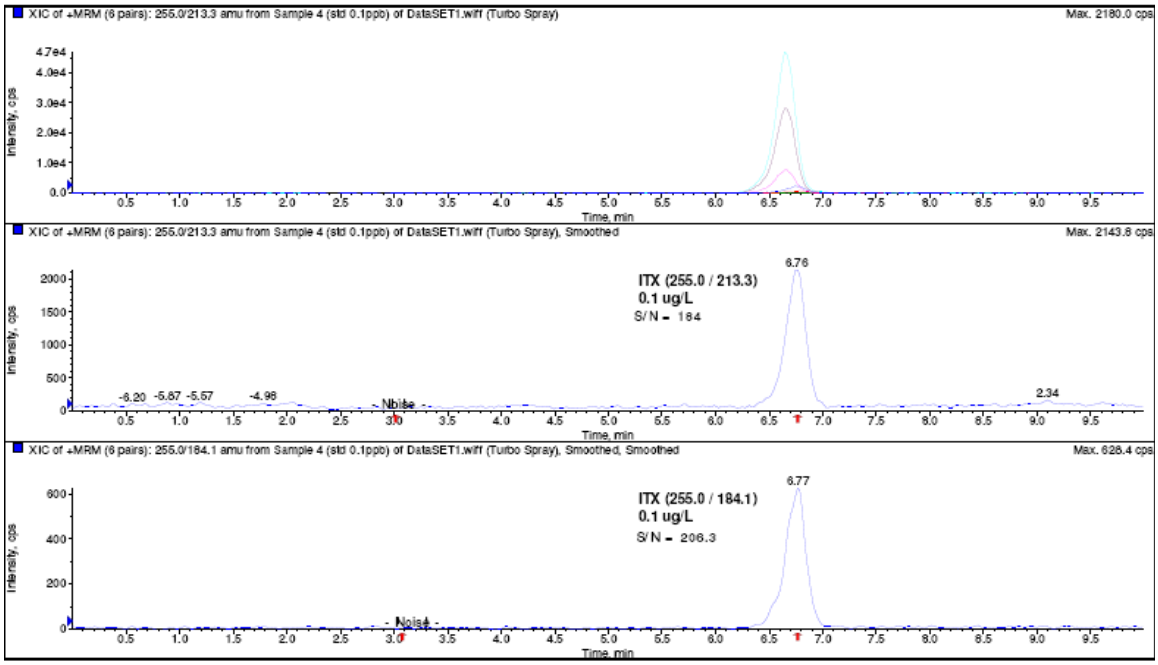

Figure 7.5 Signal- to- noise (S/N) ratio of the 2 qualifying mass pairs of ITX at 0.1 µg/L level in the MRM spectrum.

7.6.2.3 Robustness

The robustness of the method was determined using by injecting a 1.0 µg/L standard solution six times repetitively on a Thermo BDS Hypersil column using 4 different liquid-

chromatograph gradient profiles. ITX was eluted between 4.99 min to 6.89 min from these profiles, with average signal-to-noise (S/N) ratios ranging between 98.3 (14.4 % RSD) to 467 (3.65 % RSD). When the 1.0 µg/L standard solution was injected six times on another column (Inertsil® ODS-3), the retention time of ITX increased to 9.35 min and had much lower sensitivity (S/N =193). Consequently, the optimum analytical conditions for ITX reported in this paper were chosen based on sensitivity and elution time. The Thermo BDS Hypersil column was selected as the optimum stationary phase to elute ITX at a retention time of about 6.8 min, which provided an excellent S/N ratio of 501 (25.9 % RSD) at 1.0 µg/L level. Over the course of the whole project which lasted about 4 month, no significant changes in the quality of the results has been found using the chosen conditions, despite slight temperature fluctuations in the room, minor variations in the mobile phases, and instrumental drift.

7.6.3 Analysis of Food Samples

Throughout the course of the method development and analysis, it was crucial to keep background contamination to the minimum, as levels of ITX analyzed were extremely low. All possible contamination arising from the use of pipette tips, filter papers, syringe filters, plastic tubes, and solvents were checked to be free from ITX during the validation process. It was also found during this extensive investigation that filter paper contained ITX at detectable parts per billion levels. As a result, centrifuging was used in place of paper filtration in this method. Strict standards of zero background contamination for all the reagent blanks and sample blanks were also adhered to during the course of food sample analyses to ensure the

quality of these published results. The method was also subsequently assessed on milk and juice samples to ensure the suitability of the method on different matrices. Six replicates of a milk and juice sample were subjected to the described sample preparation protocol with satisfactory results (Juice: 0.72 ± 0.03 μg/kg; milk: 0.74 ± 0.04 μg/kg). Fortified samples in similar matrix were also recovered in the range of 101 % to 106 %. Qualification of ITX in these samples was made only if the concentrations of the 2 qualifying MRM mass pairs (255.0/213.3) and (255.0/184.1) in the sample were within a range of 80 % to 120 %.

A range of 39 different types of packaged beverages were later purchased locally for the analysis of ITX using the fully optimized method (Table 7.3). Detectable amounts of ITX were found in all the food packaging materials (0.004 μg/dm^2 – 108.600 μg/dm^2).

Table 7.3 Results of ITX in food and in the respective food packaging materials.

Marking	Sample Description	Result in Food (µg/kg)	Result in Packaging (µg/dm^2)
Drink 1	Original isotonic drink	< 0.5	0.197
Drink 2	Bandung drink	< 0.5	0.165
Drink 3	Rose hip drink	< 0.5	0.548
Drink 4	Cocktail drink	< 0.5	0.066
Juice 1	100% orange juice concentrate	< 0.5	0.013
Juice 2	Orange drink	< 0.5	0.012
Juice 3	Pineapple & selected fruit juice with fibre	< 0.5	0.004
Juice 4	100% pure red grape juice	< 0.5	0.004
Juice 5	Tomato juice	0.96	0.220
Juice 6	100% grapefruit & selected fruit juice blend with fruit cells	< 0.5	0.005
Juice 7	Blackcurrant juice drink	< 0.5	0.008
Juice 8	100% apple juice concentrate drink	< 0.5	0.046
Juice 9	100% orange juice	< 0.5	0.466
Juice 10	100% carrot juice	84.30	57.300
Juice 11	100% orange juice concentrate	0.53	0.716
Juice 12	Pineapple beverage	80.90	108.600
Juice 13	100% pure squeezed orange juice	< 0.5	0.180
Juice 14	Natural lemon drink with grapefruit pulp	< 0.5	0.405
Juice 15	Guava juice with pear bits	< 0.5	0.010
Juice 16	Orange juice drink	3.85	2.364
Juice 17	Orange juice concentrated drink (without sugar)	< 0.5	0.257
Juice 18	Orange fruit drink	< 0.5	0.079
Juice 19	Tomato juice	< 0.5	0.033
Milk 1	Coffee with milk	< 0.5	0.051
Milk 2	Full cream milk	< 0.5	0.026
Milk 3	Full cream milk	< 0.5	0.031
Milk 4	Soybean milk	< 0.5	0.090
Milk 5	Chocolate flavoured soy milk	4.78	0.833
Milk 6	Extra full cream milk	< 0.5	0.086
Milk 7	Soy bean milk	< 0.5	0.048
Milk 8	Fresh milk	< 0.5	0.033
Milk 9	Reduced sugar fresh soya milk	13.62	5.924
Milk 10	Low fat milk	< 0.5	0.112
Milk 11	Soy bean milk	< 0.5	0.094
Tea 1	Chrysanthemum tea	< 0.5	0.037
Tea 2	Jasmine tea	< 0.5	0.105
Tea 3	Luo Han Guo herbal tea	< 0.5	0.165
Yoghurt 1	UHT yogurt peach drink	< 0.5	1.560
Yoghurt 2	Yoghurt fruit drink	< 0.5	0.057

However, migration of ITX into foods occurred in only 7 of the 39 samples (0.53 µg/kg to 84.30 µg/kg) (Figure 7.6). These affected samples were observed to have heavy print on all sides, where the residual ITX content in the food packaging material was determined to be at

least 140 % higher than the average ITX content in the rest of the food packaging materials. Hence, this suggests that the amount of printing on the food packaging material may have an important effect on the content of photo-initiators found in the food, and was therefore a critical factor for the observed migration activity.

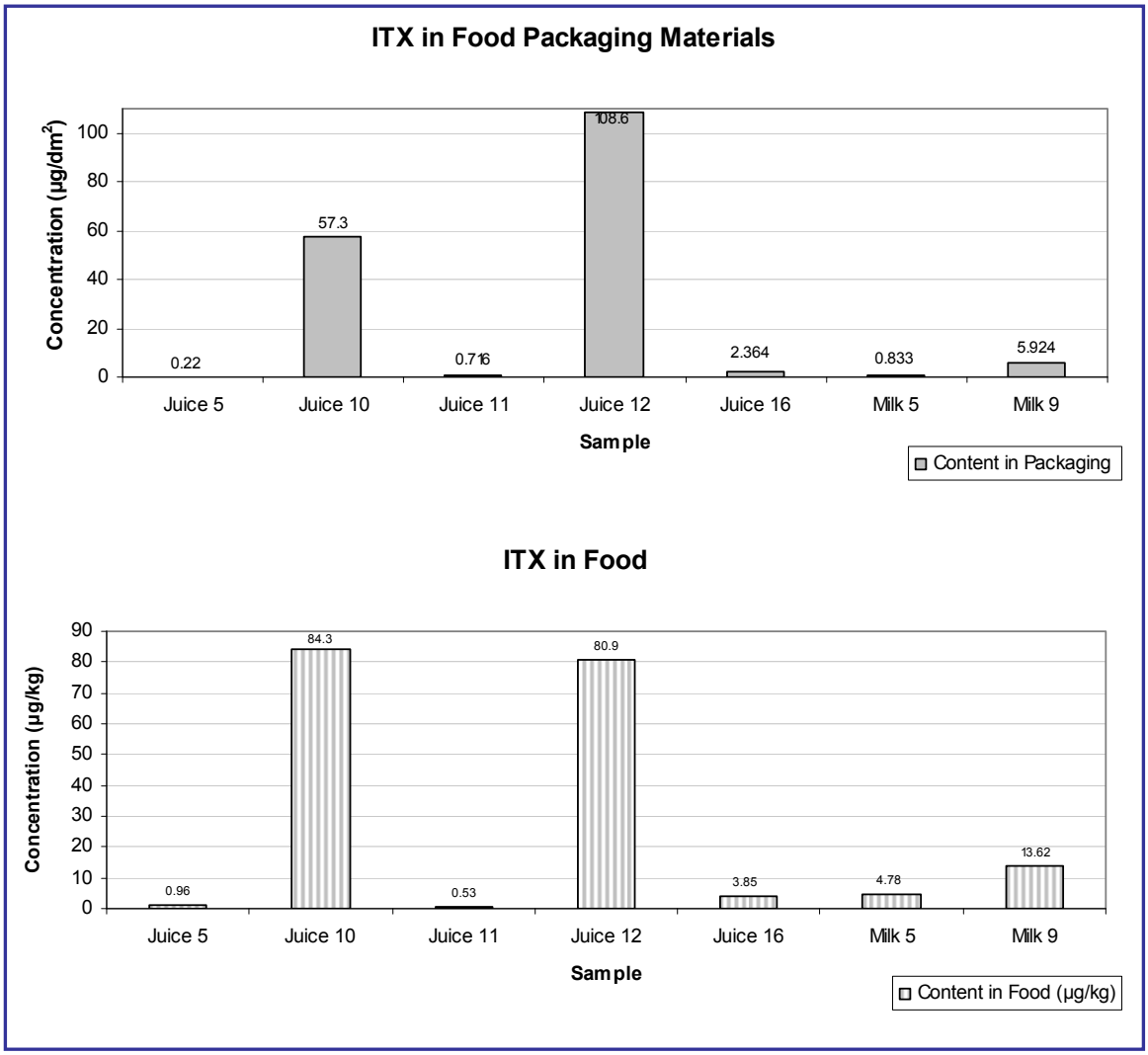

Figure 7.6 Correlation between affected samples and the residual ITX content in food packaging material.

The matrix of the food sample may also be important in affecting the migration of ITX. Being a lipophilic molecule, ITX is likely to be more soluble in milk, which contain higher fat content than juices. In a study consisting of various milk, soy beverages and juice samples presented by the European Food Safety Authority (EFSA) [10], it was reported that migration of ITX was higher into fat-containing milk and soy beverages, while clear juice samples had no detectable ITX content. However from our results, this observation was not obvious as both milk and juice samples contained detectable amounts of ITX. An explanation for this discrepancy may be attributed to the manufacturing process of these food samples. Stacking the sheets of printed food packaging material one on top of the other prior to cutting them into shapes required for food packaging is common in such packaging premises. The stacking of the printed material may have transferred residual ITX from the external printed side to the above interior (non-printed) side on contact [11], and as a result, the ease of ITX migrating from the packaging material into food is increased, which is independent of the food matrix.

7.7 Conclusions

This method has been shown to be suitable for detecting low levels of ITX in a number of matrixes ranging from acidic juices to fatty milk and yoghurt drink samples with excellent recoveries at low parts per billion levels. Extremely good validation data on precision, accuracy, linearity and robustness have been obtained, which enhances the confidence of using the established protocol on these different matrixes. The ITX content analysed in the range of food packaging materials ranged between 0.004 μg/dm^2 to 108.600 μg/dm^2, and

between < 0.50 to 84.30 μg/kg in food. The detectable amounts of ITX in the beverages may have resulted from the migration of ITX from the external printed side of the food packaging material, or from the contamination of ITX due to the stacking of the sheets of printed packaging material prior to cutting and shaping them for the beverage – filling process. The low MDL of 0.15 μg/kg and MQL of 0.50 μg/kg established in this simple method allow the enforcement of the specific migration limit of 50 μg/kg as imposed by the Bundesinsitit fuer Risikobewertung (BfR), and therefore provides utility for both food producers and food safety surveillance institutions.

7.8 References

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CHAPTER 8

Chapter 8

Measurement Uncertainty of Isopropyl-9H-thioxanthen-9-one in Packaged Beverages by Solid Phase Extraction Clean-up and Liquid Chromatography with Tandem Mass Spectrometry Detection

8.1 Introduction

Method validation is an important requirement in the practice of analytical chemistry. It is the process of defining an analytical requirement, and confirming that the method under consideration has performance characteristics and capabilities consistent with what the application requires. In other words, it determines the fitness of purpose of the intended analysis. Methods need to be validated in the following circumstances: when an established method has been revised to incorporate improvements or extended to a new problem; when a new method has been developed for a particular problem; when quality control indicates that the established method has been changing with time; or when an established method is to be used in a different laboratory, with different analysts, or with different instrumentation(s) [1].

All analytical measurements have measurement errors, which can be attributed to gross errors (blunders), systematic errors, or random errors. Hence, it is important to evaluate possible errors in the estimated analytical value, as provided by the test results, a process also known

as the uncertainty measurement. This ensures that the analytical data is technically sound and defensible, especially when there are cases of disputes between inter-laboratories, and also allow for the improvement of confidence associated with the reliability of analytical results of food samples, that can allow for the removal of barriers to international trade.

The uncertainty is usually not due primarily to the errors by the analysts. Rather, it is inherent in the measuring process. The key principles of method validation and uncertainty estimation requires that the validation studies to be representative of the normal operation of the method. In addition, the studies must encompass the complete method which includes the representative range of sample matrices, as well as the representative linear range of the analyte(s). Therefore, an accurate estimation of the measurement uncertainty would require the combination of the estimations of precision, trueness (bias), as well as other uncertainty contributions such as the purity of certified reference standards, data from recognized proficiency programmes, as well as any sub-sampling errors, that could possibly contribute to the accuracy of the analytical result.

Measurement Uncertainty = precision + bias + others **(Equation 8.1)**

In this work on ITX, we estimate the measurement uncertainty based on the precision data, bias, as well as the uncertainties associated with the purities of the ITX and d_7-ITX standards, since these factors are more relevant in this context with respect to the rest of the parameters described earlier. The following paragraphs therefore illustrates the process of estimating the precision, accuracy, as well as other sources of uncertainties of the method to compute and

determine the measurement uncertainty of ITX content in beverages in this method, using solid-phase extraction clean-up and LC Tandem MS, as described in greater detail in Chapter 7.

8.2 Precision study (Inter-day)

Precision refers to the closeness of the agreement between the results of successive measurements of the same analyte carried out under the same conditions of measurement. The inter-day precision of ITX was estimated through the repeated analysis (n = 10) of the same standard solution at 0.1 ng/L over 3 days, using the same instrument, with the objective of determining the standard uncertainty due to run- to- run variation of the overall analytical process over 3 days (Table 8.1).

Table 8.1 Inter-day Precision study for ITX

Sample	ITX Peak Area (counts)		
	Day 1	**Day 2**	**Day 3**
Standard-1	5.68×10^3	5.74×10^3	5.73×10^3
Standard-2	5.70×10^3	5.69×10^3	5.77×10^3
Standard-3	5.64×10^3	5.75×10^3	5.76×10^3
Standard-4	5.56×10^3	5.67×10^3	5.67×10^3
Standard-5	5.58×10^3	5.67×10^3	5.76×10^3
Standard-6	5.68×10^3	5.72×10^3	5.68×10^3
Standard-7	5.74×10^3	5.67×10^3	5.75×10^3
Standard-8	5.68×10^3	5.69×10^3	5.71×10^3
Standard-9	5.63×10^3	5.69×10^3	5.71×10^3
Standard-10	5.67×10^3	5.68×10^3	5.74×10^3
Mean Peak Area of standard peak on day of study (counts)	5656	5697	5728
Standard deviation	54.61	29.46	34.58
Coefficient of variation (CV)	**0.00966**	0.00517	0.00604

In order to estimate the worst case uncertainty with respect to precision for this project, we use the largest coefficient of variation of the first day for the computation of the measurement uncertainty of the overall process (Table 8.1). The uncertainty of inter-day precision was therefore found to be 0.00966.

8.3 Bias Study

Bias refers to the difference between the expectation of the test results with an accepted reference value. The bias of the analytical procedure was investigated during the in-house validation study using spiked liquid milk samples. Table 8.2 provides the results of an extensive study of spiked liquid milk samples (n = 8) at each concentration level: 0.5, 20, and 100 ng/g.

Table 8.2 Results of recovery (bias) study at 0.5 ng/g, 20 ng/g and 100 ng/g levels.

	Recovery %		
	0.5 ng/g level	**20 ng/g level**	**100 ng/g level**
Milk-1	97.02	98.39	99.48
Milk-2	99.62	100.39	99.08
Milk-3	97.42	99.89	98.68
Milk-4	98.42	99.89	97.68
Milk-5	103.02	101.89	99.18
Milk-6	102.62	102.89	97.88
Milk-7	98.42	102.39	98.98
Milk-8	103.02	102.39	100.88
Mean recovery %	99.95	101.02	98.98
Standard Deviation	2.56	1.60	0.99
Coefficient of variation (CV)	0.0256	0.0158	0.0100
RSD %	2.56	1.58	1.00

Standard uncertainty U(x) at 0.5 ng/g level = $\frac{sd}{\sqrt{n}} = \frac{0.0256}{\sqrt{8}} = 0.00905$

Standard uncertainty U(x) at 20 ng/g level = $\frac{sd}{\sqrt{n}} = \frac{0.0158}{\sqrt{8}} = 0.00559$

Standard uncertainty U(x) at 100 ng/g level = $\frac{sd}{\sqrt{n}} = \frac{0.0100}{\sqrt{8}} = 0.00354$

8.3.1 Calculation of bias based on recovery data

In a perfect situation, the recovery (R) would be exactly unity (1) but in reality, circumstances such as imperfect sample extractions, standard calibration and purity may result in observations that differ from the ideal. We can determine the recovery for any significant departure from unity by using the Students' *t*-test, by considering the question:

"Is $|R - 1|$ greater than u_R, the uncertainty in the determination of R ?"

The significance testing can therefore be conducted as follows:

$H_0 : | R - 1| / u_R < t_c$ *R does not differ significantly from 1*

$H_1 : | R - 1| / u_R > t_c$ *R differs significantly from 1*

where *t* is the critical value [2].

To calculate the bias based on the available recovery data from the ITX experiments, the student's t - distribution significance test was subsequently taken to test if the recoveries at 3 concentration levels: 0.5, 20, and 100 ng/g, were significantly different from 1. For 7 degrees

of freedom (n = 8), t_c = 2.365 according to the two-tailed critical t_α values of Students' t variables tabulated in Table 8.3.

Table 8.3 Two-tailed critical t_α values of Students' t variables at 95 % Confidence intervals.

Degrees of freedom	Critical t_α value at 95 % confidence interval
1	12.706
2	4.303
3	3.182
4	2.776
5	2.571
6	2.447
7	**2.365**
8	2.306
9	2.262
10	2.228

8.3.1.1 $t_{0.5} = \frac{|1-Recovery|}{U(x)} = \frac{|1-0.9995|}{0.00905} = 0.055$

8.3.1.2 $t_{20} = \frac{|1-Recovery|}{U(x)} = \frac{|1-1.0102|}{0.00559} = 1.825$

8.3.1.3 $t_{100} = \frac{|1-Recovery|}{U(x)} = \frac{|1-0.9898|}{0.00354} = 2.881$

From the above calculations, $t_{0.5}$ and t_{20} values were less than the critical value, t_c = 2.365, therefore, it indicates that recoveries at 0.5 and 20 ng/g were not significantly different from 1. However, the value of t_{100} is greater than the corresponding critical value, t_c = 2.365, the

recovery would be significantly different from 1, but in the normal application of the method, no corrections were applied to the analytical results as the recoveries were usually well within 90 – 110 %. The uncertainty must be increased to take account of the fact that the recovery had not been corrected for. The increased uncertainty (U_i), is therefore given by:

$$U_{i(100)} = \sqrt{\left(\frac{1-\text{Recovery}}{k}\right)^2 + (U(x))^2} = \sqrt{\left(\frac{1-0.9898}{2}\right)^2 + (0.00354)^2} = 0.00620$$

To estimate the worst case scenario for the estimation of the measurement uncertainty, we use the largest increased uncertainty, U_i (x) at 0.5 ng/g level :

$$U_{i(0.5)} = \sqrt{\left(\frac{1-\text{Recovery}}{k}\right)^2 + (U(x))^2} = \sqrt{\left(\frac{1-0.9995}{2}\right)^2 + (0.00905)^2} = 0.00905$$

Therefore, the uncertainty for bias was calculated as 0.00905.

8.4 Other Sources of Uncertainty

8.4.1 Balances/ Volumetric Measuring Devices

All balances and the important measuring devices were under regular control.

Precision and recovery studies have already taken into account the influence of the calibrations of the different volumetric measuring devices, such as volumetric flasks and pipettes, which were calibrated prior to use.

8.4.2 Sample Homogeneity

The beverage samples were shaken for 20 min prior to sample preparation on the rotating platform, and were assumed to be homogeneous.

8.4.3 Reference material purity

8.4.3.1 ITX standard

The purity given by the manufacturer was 97 % [98.5 ± 1.5 %].

Assuming a rectangular distribution, the purity of the ITX standard had a standard uncertainty of : $\frac{0.015}{\sqrt{3}} = 0.00866$

8.4.3.2 d_7-ITX internal standard

The purity given by the manufacturer was 99.5 % [99.75 ± 0.25 %].

Assuming a rectangular distribution, the purity of the d_7-ITX had a standard uncertainty of:

$$\frac{0.0025}{\sqrt{3}} = 0.00144$$

8.5 Summary of Uncertainty Estimation of ITX

Table 8.4 Overall uncertainty estimation of ITX procedure

	Description	Value x	Standard uncertainty u(x)	Relative Standard uncertainty u(x)/x	Comments
A	Precision (inter-day)	1	0.00966	0.00966	Evaluation of precision of instrument during repeated injections of a 0.1 μg/L standard over a period of 3 days. The worst case scenario of Day 1 precision CV was used for the computation of ITX uncertainty.
B	Bias	0.9995	0.00905	0.00905	Evaluation of accuracy of spiked samples at 3 concentration levels: 0.5 ng/g, 20 ng/g, 100 ng/g
C_1	Purity of ITX standard	0.97	0.00866	0.00893	As per certificate of analysis
C_2	Purity of d_7-ITX standard	0.995	0.00144	0.00145	As per certificate of analysis
	$U(P_{op})/ P_{op}$	**--**	**--**	**0.0160**	

Combined uncertainty =

$$\sqrt{(A)^2 + (B)^2 + (C_1)^2 + (C_2)^2} = \sqrt{(0.00966)^2 + (0.00905)^2 + (0.00893)^2 + (0.00145)^2}$$

= **0.0160**

Table 8.4 illustrates the various estimations of uncertainty components associated with the overall ITX procedure. The expanded uncertainty U (P_{op}) of the overall procedure at 95% confidence level is obtained by multiplying the combined standard uncertainty with a coverage factor (k) of 2, giving 0.032, or about 3.2 %. The value obtained is a rough estimate of the combined measurement uncertainty. The accurate measurement uncertainty budgeting, as stated in [3], is beyond the scope of this thesis.

8.6 Conclusions

The measurement uncertainty of the ITX in packaged beverages by solid phase extraction clean-up and liquid chromatography with tandem mass spectrometry detection had been estimated, and the expanded uncertainty estimate was only about 3.2 % of the analytical result.

The small uncertainty measurement reflected well on the validated method, and provided reliability on the use of this proposed method for analyzing the photoinitiator content in the wide range of beverages.

8.7 References

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CHAPTER 9

Chapter 9

Determination of Benzophenone, isopropyl-9H-thioxanthen-9-one, thioxanthen-9-one, 2, 4-dimethylthioxanthone, 2-chlorothioxanthen-9-one in Packaged Beverages by Solid Phase Extraction Clean-up and Liquid Chromatography with Tandem Mass Spectrometry Detection

9.1 Introduction

Traditionally, printing inks were thermally cured, and the common formulations included organic solvents or water. However, with increasing environmental concerns on the usage and production of volatile organic compounds (VOCs), and demands for more efficient production processes, ultraviolet (UV) -cured inks were developed. In UV-cured inks, photoinitiators were incorporated into the ink formulations to cure inks for use on printing paper and cardboard, via the initiation by the photoinitiators to bring about radical polymerization of the acrylic components and oligomers (Figure 9.1) in the UV inks [1].

UV-cured inks typically contain about 5-10 per cent of photoinitiator content. However, only a small proportion of the initiators are used up during the curing process. The unreacted photoinitiators can therefore remain in the printed material, and potentially migrate into food when used on printed food packaging materials. The use of UV-cured inks for printing cartonboard have become widespread because the fast cure permits on-line cutting and folding, enabling rapid production of finished packaging.

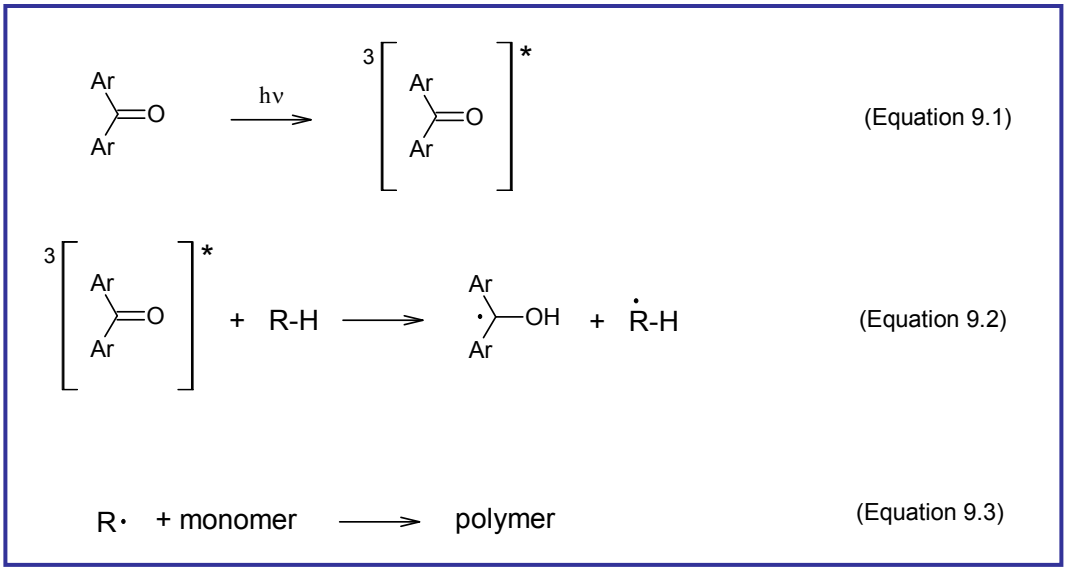

Figure 9.1 Polymerisation reaction mechanism of photoinitiators [2]

Currently, photoinitiators in ink, unlike many other substances that come into contact with food, are not regulated by specific European legislation. However, materials intended to come into contact with food should comply with the general criteria laid down in Article 3 of Regulation (EC) No. 1935/2004, stating that materials and articles in contact with food should not transfer their constituents to food in quantities which could change the composition of the food or bring about unacceptable deterioration in the organoleptic characteristics thereof [3]. According to European Food Safety Authority (EFSA), although the photoinitiator, ITX had not shown any adverse health effects after consumption by the public, its presence in food is still considered as undesirable as studies have found that it may possibly have strong interactions with the lipid moiety with the biological membranes, and therefore affect the phospholipids organization in biological membranes [4]. Besides that, the US Environment Protection Agency (EPA) has regarded the ITX as a potential hazard for human health and

environment at a lower concentrations than those found in packaged milk and other drinks [5]. Therefore, a specific migration limit (SML) of 0.05 mg/kg had been deduced by the Federal Institute for Risk Assessment (BfR) [6].

Besides ITX, residues of other photoinitiators such as benzophenone (BP) have been found in food samples and their relative packaging material [7,8]. In a particular study on BP migration from its relative packaging material to food indicates that 72 % of the 71 samples studied were contaminated by this compound in the range of 0.01– 7.3 mg/kg [9]. Therefore, a specific migration limit (SML) of 600 μg/L was set on BP by the European Union to state the maximum amount of BP which is permitted to migrate from packaging material to the food [10]. A tolerable daily intake (TDI) of 0.01 mg/kg based on bodyweight for BP was also set by the European Commission Scientific Committee on Food [11].

At present, there are very limited analytical methods to detect photoinitiators in food and in their packaging materials, and even fewer papers for the simultaneous analytical determination of a range of photoinitiators from printed food packaging material or packaged foods. Morlock et al. [12] published a novel methodology that made use of the accelerated solvent extraction (ASE) system for the extraction of the ITX from milk, yoghurt and fat and utilizing high performance thin layer chromatography coupled with mass spectrometer (HPTLC-MS) for detection. Although this extraction method was relatively simple and rapid, improvements on the mean recovery of ITX could be made. Most other relevant methods available in literature utilized GC-MS or HPLC/diode-array detection (HPLC-DAD) for the detection of photoinitiators [13,14].

The objective of this research was to develop, optimize and validate a simple analytical methodology using LC-MS/MS for the simultaneous determination of a range of five photoinitiators in food, as well as their cartonboard packaging material. This reported methodology in this chapter is an improvement to the earlier described ITX methodology, as described in Chapter 8. The photoinitiators that were analyzed are: benzophenone (BP), isopropyl-9H-thioxanthen-9-one (ITX), thioxanthen-9-one (TX), 2, 4-dimethylthioxanthone (DMTX), 2-chlorothioxanthen-9-one (CTX). The mass spectrometry method also utilized two internal standards, namely, 1-hydroxycyclohexyl-phenylketone (1-HCPK) and 2-Isopropyl-$2H_7$-thioxanthen-9-one (ITX-d_7). The developed and validated method was subsequently tested on real food beverages and the respective food packaging to test the suitability of this optimized method on various food matrices such as acidic fruit juices and milk.

9.2 Experimental

9.2.1 Materials and Reagents

Benzophenone (molecular weight 182 D), isopropyl-9H-thioxanthen-9-one (molecular weight 254 D, 97% mixture of 2-and 4-isomers), thioxanthone (molecular weight 212 D), 2-Chlorothioxanthone (molecular weight 246 D), 2,4-dimethylthioxanthone (molecular weight 164 D) were purchased from Sigma-Aldrich. The internal standards, 1-hydroxycyclohexyl-phenylketone (1-HCPK, molecular weight 204 D) was purchased from Sigma-Aldrich and 2-

Isopropyl-2D_7-thioxanthen-9-one (molecular weight 261 D) was purchased from Witega Laboratorien (Berlin, Germany).

Acetonitrile, isooctane, and methanol were HPLC grade and supplied by Labscan Asia (Bangkok, Thailand). Formic acid was supplied by Kanto Chemical Co. Inc. Deionized water was purified by the Elga PureLab Ultra water system (High Wycombe, UK) and was filtered through a Millipore filtration system (Billerica, MA, USA) with a filter pore-size of 0.45 μm prior to use. Carrez reagent I (potassium hexacyanoferrate (II) trihydrate) was purchased from Sigma-Aldrich and Carrez reagent II (zinc acetate) was purchased from Perkin-Elmer; Waltham, MA, USA. The Oasis HLB cartridges (60 mg, 3 mL) were purchased from Waters (Milford, MA, USA) to perform solid phase extraction on food beverages.

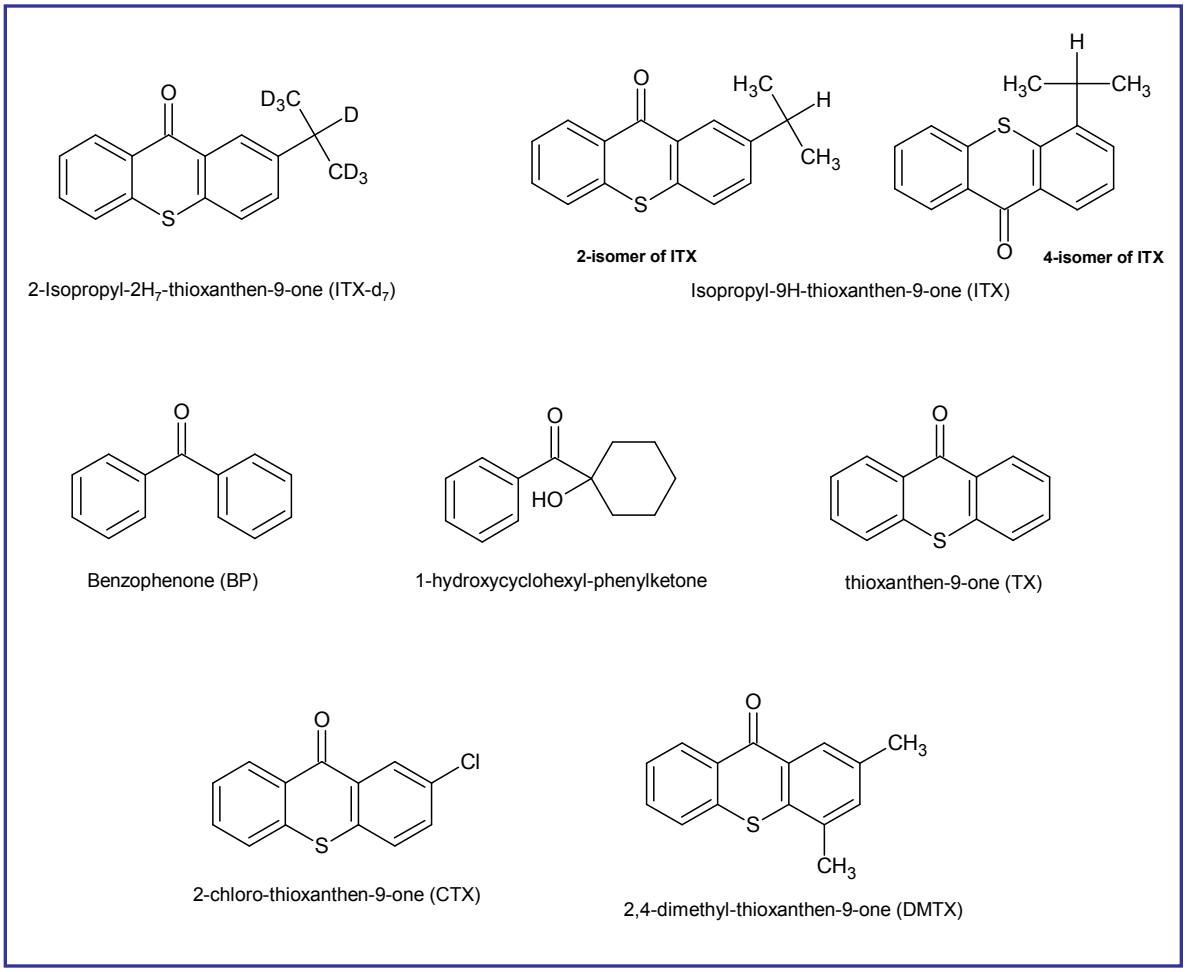

Figure 9.2 Chemical structures of all the photoinitiators in this study

Primary stock solutions (100 mg/L) of individual photoinitiators (BP, ITX, 1-HCPK, ITX-d_7) were prepared using acetonitrile. Stock standards TX, DMTX and CTX (dissolved in isooctane) were blown dry in their respective volumetric flasks using a gentle stream of nitrogen gas, and redissolved in acetonitrile to yield TX at a concentration of 55, and 110 mg/L for both CTX and DMTX. Intermediate individual solutions (10 mg/L) and internal standards (1-HCPK and ITX-d_7; 1 mg/L) were prepared by diluting the respective primary stock solutions using acetonitrile. A mixed standard solution (10 mg/L) containing the five photoinitiators (BP, ITX, TX, DMTX and CTX; Figure 9.2) was subsequently prepared in a 10 mL volumetric flask and topped up using acetonitrile. The intermediate mixed standard

solution (1 mg/L) was prepared by diluting the 10 mg/L mixed standard solution appropriately with acetonitrile.

All primary and immediate stock solutions were stored in the refrigerator at 5 °C for no longer than three months. All working standards were prepared fresh when needed.

9.3 Apparatus

LC-MS/MS analyses were performed using an Agilent 1100 LC system coupled to an API 3000 Q-Trap LC-MS/MS with TurboIon Spray source (Applied Biosystems, Foster City, CA, USA). LC injection volume: 25 µL; run length: 15 min; column temperature: 40 ^{o}C; column: Inertsil HPLC column (Ph-3, 3 µm, 150 mm x 3 mm I.D. (G.L. Sciences, Tokyo, Japan); flow rate: 300 µL/ min, where 0 – 2 min: 50: 50 A: B (v/v); 2 min: 85: 15 A: B (v/v), 6 min: 95: 5 A: B (v/v); 10 - 15 min: 50: 50 A: B (v/v), where A = methanol, and B = filtered 0.1 % formic acid solution. Prior to each series of chromatographic separations, the analytical column was conditioned for 15 min with methanol, and equilibrated with methanol : 0.1 % formic acid solution (50: 50, v/v) for at least 15 min to provide a stable baseline for subsequent chromatographic analysis. The mass spectrometer (MS) was carried out using the multiple reaction monitoring (MRM) mode, and was equipped with an electrospray ionisation (ESI) interface operating in the positive mode. MS source temperature: 400 °C; ion spray: 5000 V. The mass spectrometry conditions are listed in Table 9.1.

Table 9.1 Mass spectrometry settings used in the optimized method.

Analyte	Precursor ion (m/z)	Product ion (m/z)	DP (V)	CE (V)	CXP (V)	FP (V)	EP (V)
BP	183.2	105.1	54	21	11	100	10
		77.0	52	48	11	100	10
ITX	255.3	213.1	47	30	9	90	7
		184.2	47	47	8	90	7
TX	213.2	184.2	23	40	2	80	10
		152.3	23	53	11	80	10
CTX	247.2	211.9	65	35	2	200	10
		184.0	65	49	7	200	10
DMTX	269.3	240.9	57	33	2	100	10
		212.9	57	45	7	100	10
HCPK	205.3	187.2	15	8	13	80	4
		105.0	16	18	9	80	4
ITX-d_7	262.4	214.5	47	30	10	90	7
		185.4	47	56	9	90	7

9.4 Samples

Two types of beverages consisting of milk and fruit juices for the validation study were obtained locally. These samples were chosen as their primary food packaging material had moderate to heavy printing on the surface. For each analytical run, a sample blank as well as a fortified milk and/or juice sample was prepared to estimate the degree of recovery. The fortified sample was prepared by gently evaporating a small volume of the mixed intermediate standard solution containing the photoinitiators and their respective internal standards accurately into a round bottom flask with a micropipette, using nitrogen gas. This fortified

sample was later subjected to the described sample preparation method below. In order to ensure accurate analytical results, all reagents used in the analysis were prepared fresh and analyzed separately to ensure that they were free from any interfering contaminants.

9.5 Sample Preparation

Food - After 500 μL of both 1-HCPK and ITX-d_7 (10 mg/L) internal standard solutions have been spiked into a flat round bottomed flask, the solvent was evaporated to dryness using nitrogen gas. Ten grams of sample was weighed into the vessel, and 100 mL of acetonitrile : water containing 1 % (v/v) of Carrez reagent 1 and 1 % (v/v) of Carrez reagent 2 (70: 30, v/v), measured in a 100 mL standard flask, was transferred to the sample. The resulting mixture was shaken for 20 min, and transferred to a centrifuge tube to be centrifuged at 3500 rpm for 15 min. Ten millilitres of the supernatant solution was pipetted out, and diluted to 30 mL with deionised water. Six millilitres of the diluted sample was loaded onto the SPE cartridge that was previously conditioned with 3 mL of methanol and equilibrated with 3 mL of water. After washing the cartridge with 3 mL of water and 3 mL of acetonitrile: water (10: 90 v/v), the analytes were eluted with 4 mL of acetonitrile. The resulting eluate was blown dry with nitrogen, reconstituted with 1 mL of acetonitrile: 0.1 % formic acid (70: 30, v/v) acetonitrile: water, and filtered through 0.45 μm nylon filters into HPLC vials prior to LC-MS/MS analysis.

Food packaging material - 200 mL of acetonitrile was added to a glass bottle containing cut pieces (of approximately 2 cm by 2 cm) of the food packaging material that were in contact with the food to extract the residual photoinitiators. The contents were soaked in the capped glass bottle for 24 h. One mililitre of the aliquot was pipetted into a HPLC vial after shaking the total contents in the glass bottle for 5 min. The extract was blown to dryness using nitrogen gas, and reconstituted with 800 μL of acetonitrile : 0.1 % formic acid (70:30 v/v), and 100 μL each of both internal standards (1 mg/L).

9.6 Results and Discussion

9.6.1 Optimization of Sample Preparation – Extraction Solvent

The use of an extraction solvent was necessary to extract the photoinitiators from the complex food samples prior to clean-up via the solid phase extraction (SPE) system. The extraction solvent consisted of acetonitrile, and deionised water containing 1 % of Carrez reagents 1 and 2. Acetonitrile was selected due to its organic nature and its similar polarity to extract the photoinitiators from the food sample, while Carrez reagents 1 and 2 were used to precipitate out, and separate any proteins present in the food matrix.

The fortified fruit juice for optimization studies were prepared by pipetting 500 μL of a solution of a mixture (10 mg/L) of the 5 photoinitiators into a round bottom flask containing 500 μL of each individual internal standard and gently evaporating the organic solvent using a

stream of nitrogen. Ten grams of the fruit juice was then weighed into the same round bottom flask for recovery studies. Prior to the optimization study, the fruit juice used for fortification was analyzed and found to contain trace amounts of BP. Thus, the results of the fortified fruit juice were corrected for any background levels for this analysis. The RSD was calculated by dividing the standard deviation by the mean, and the value multiplied by 100 %.

Table 9.2 Recovery of photoinitiators, at different composition of extracting solvent (deionised water containing 1 % of Carrez reagents 1 and 2) : acetonitrile, v/v.

Analyte	Recovery of photoinitiators in apple juice with different extracting solvent composition [(deionised water containing 1 % of Carrez reagents 1 and 2) : acetonitrile, (v/v)]									
	35 : 65		30 : 70		25 : 75		20 : 80		15 : 85	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
BP	19.31	1.46	28.86	1.72	21.91	1.29	10.56	4.68	10.28	2.05
ITX	64.85	1.42	69.30	0.61	67.50	0.63	67.20	0.42	62.85	0.79
TX	76.80	1.66	80.25	1.50	78.55	0.99	73.80	2.11	62.35	0.57
DMTX	49.55	0.71	69.60	0.61	60.95	0.81	54.35	1.43	56.85	1.87
CTX	65.95	2.68	69.80	1.22	68.50	1.24	66.50	0.21	61.70	0.69

The results in Table 9.2 indicate that the recovery increases as the composition of the extracting solvent changes from 35 : 65, (v/v) to 30: 70, (v/v). However, a decreasing trend for the recovery was exhibited when the composition of the extracting solvent was varied from 25 : 75, to 15 : 85 (v/v). This decreasing effect was most evident for BP. Hence, the

optimized proportion of deionised water containing 1 % of Carrez reagent 1 and 2 : acetonitrile in the extraction solvent was chosen to be 30 : 70 (v/v). The relatively small variation results (RSD %: 0.57 % to 2.68 %) in Table 9.2, obtained from the repetition analysis (n=2) also indicated favourably on the reliability of the results.

9.6.2 Optimisation of SPE Protocol – Wash Solvent

9.6.2.1 Deionised Water

Solid phase extraction methods require a washing procedure to remove unwanted impurities which may otherwise interfere with the analytical results. The volume of the wash solvent selected must be able to remove the unwanted impurities, yet at the same time, retain the analytes inside, within the sorbent of the SPE cartridge. This is one reason why the polarity of the wash solvent chosen has critical consequences on the final analytical result(s). Therefore, effort has been put into this part of the research to enhance the recovery of the analytes by optimizing both the type of solvent, as well as the volume of solvent, in order to achieve the best results. Table 9.3 illustrates the mean recovery of analytes from the protocol after varying the amount of water used as the wash solvent.

Table 9.3 Recovery of analytes after varying the amount of wash solvent (water)

Analyte	Water - 3 mL		Water - 1 mL	
	Mean Recovery (%)	RSD (%)	Mean Recovery (%)	RSD (%)
BP	28.86	1.72	27.35	1.30
ITX	69.30	0.61	71.45	0.89
TX	80.25	1.50	82.90	1.02
DMTX	69.60	0.61	67.05	2.64
CTX	69.80	1.22	72.10	0.78

From the results, the recoveries were comparable between different amounts of the wash solvent used. This indicates that the quantity of deionised water as the washing solvent did not hamper the recovery. However, 3 mL of deionised water were chosen as it was difficult to predict whether the presence of water soluble impurities in various food matrixes will interfere with the result.

9.6.2.2 Acetonitrile: Deionised Water

The less than ideal recoveries listed in Table 9.3 suggest that using only water to rinse out the interferences from the SPE cartridge may be insufficient to remove the presence of all unwanted interferences from the sample matrix. A secondary wash solvent involving an organic mixture of acetonitrile and water was therefore chosen to be incorporated into the

procedure. Table 9.4 illustrates the recoveries of all the analytes after an additional wash step involving different proportions of acetonitrile, being the organic solvent, was incorporated.

Table 9.4 Recoveries of analytes after incorporating an additional step of different proportions of acetonitrile : deionised water, v/v.

Analyte	5 : 95		10 : 90		15 : 85		20 : 80		25 :75	
	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)	Mean (%)	RSD (%)
BP	43.16	1.66	40.81	0.84	35.89	1.39	27.86	0.49	22.49	2.19
ITX	102.50	2.07	101.00	1.40	80.00	0.88	70.00	1.21	63.05	3.03
TX	111.50	1.90	112.50	0.63	93.65	0.83	81.30	1.04	77.80	1.64
DMTX	85.15	0.75	83.60	1.69	73.05	0.87	70.10	0.81	63.20	0.90
CTX	87.90	1.29	87.35	2.35	74.90	1.13	70.50	1.40	62.45	2.15

The results in Table 9.4 indicated that the recoveries from using 5:95 (v/v) and 10:90 (v/v) of acetonitrile: water, were most efficient in removing the organic interferences. However, it could be seen that as the proportion of organic solvent increased, the recoveries of the analytes dropped, which was indicative of the increasing solubility of the analytes with the increasing amounts of organic solvent used as the wash solvent. In view of selecting the best solvent to rinse away all unwanted interferences, while retaining the analytes within the sorbent of the SPE cartridge, 10 : 90 (v/v) of acetonitrile : water (v/v) was chosen as the optimum secondary organic solvent to wash the organic interferences from the sample matrix without hindering the recovery of the analytes.

9.6.3 Optimization of Mobile Phase Gradient

The mobile phase gradient was optimized to improve upon the analytical run time. In the beginning, the entire run time taken was relatively long at 21 minutes, although the elution time for the slowest eluting analyte was well within 13 minutes. This could be further improved by modifying the mobile phase gradient to incorporate a starting gradient with a higher percentage of organic content. Table 9.5 illustrates the modifications made to the mobile phase gradient, and the respective run time(s).

Table 9.5 Mobile phase gradient and the respective analytical run time conditions.

Before Optimization			After Optimization		
Time (min)	**Methanol (%)**	**0.1 % Formic Acid (%)**	**Methanol (%)**	**0.1 % Formic Acid (%)**	**Time (min)**
0	20	80	50	50	0
1	20	80	85	15	2
5	5	95	95	5	6
10	20	80	50	50	10
21	20	80	50	50	15

After optimization, the run time was sharply reduced by more than 25 % from 21 min to 15 min, while the elution time for all the analytes was reduced to 11.5 min (Figure 9.3). With this reduction in analytical run-time, it has resulted in greater efficiency for the overall throughput, and cost savings in terms of the mobile phase usage, as well as operating costs. The reduction in the usage of organic mobile phase usage also allowed for a greener environment, since less volume of organic solvent needs to be disposed after the analysis. As a result, the sample

throughput increased significantly by the modifications to the mobile phase gradient which has led to a higher sample volume throughput that is important for commercial laboratories.

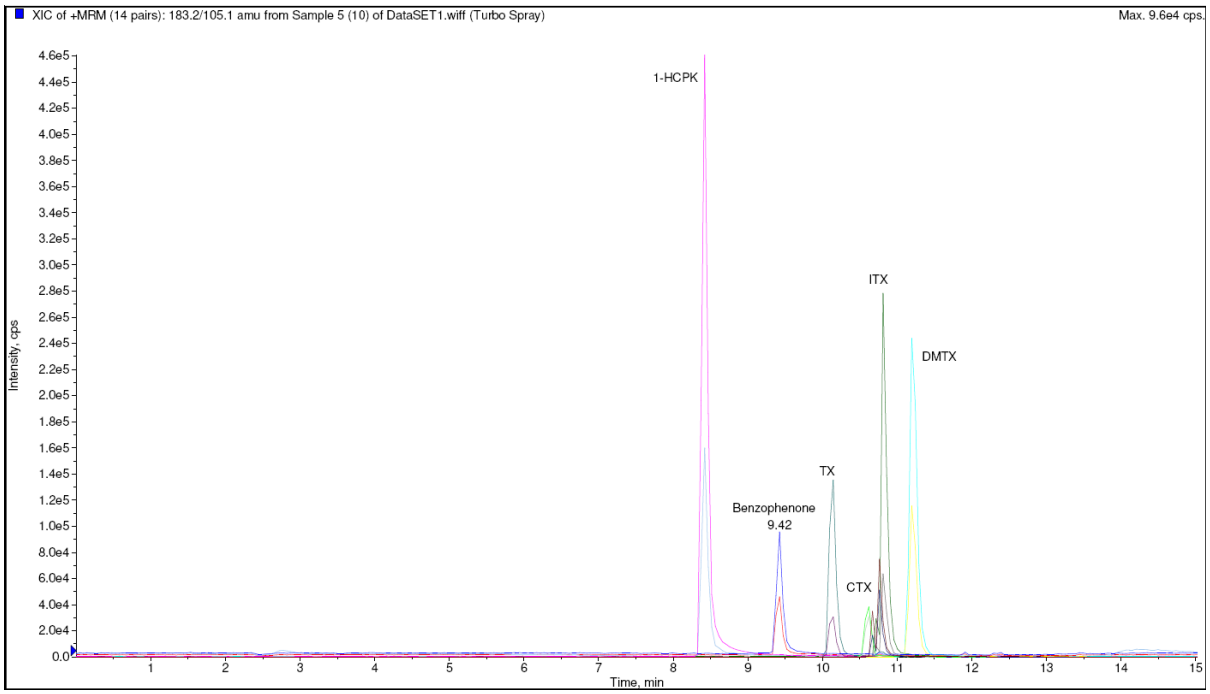

Figure 9.3 Chromatogram of a 10 ng/L standard solution containing the five photoinitiators studied.

9.6.4 Method Validation

9.6.4.1 Linearity. Method Detection Limit (MDL) and Method Quantification Limit (MQL)

Linearity was evaluated using calibration plots of peak area as a function of the analyte concentration, with the aid of a regression line by the method of least-squares, using concentration levels of 10, 20, 50, 100, 200, and 500 μg/L. Excellent correlation coefficients

(> 0.999) were obtained from the determination of six repetitions of calibration curves of the various analytes (Table 9.6).

Table 9.6 Linearity of various photoinitiators (n=6)

Analyte	Mean Correlation Coefficient (n=6)	Standard Deviation	RSD (%)
BP	0.9995	0.0004	0.0373
ITX	0.9994	0.0003	0.0267
TX	0.9993	0.0004	0.0377
DMTX	0.9992	0.0002	0.0198
CTX	0.9992	0.0002	0.0151

The method detection limit(s) for the various photoinitiators were determined by analyzing a series of fortified food samples (fruit juice and milk) spiked in the range of 10, 20, 50, 100, 200, and 500 μg/L, and determined as 3 times the signal-to-noise ratio (Table 9.7). The method quantification limit was taken as 10 times the signal- to- noise ratio. Figure 9.4 illustrates the spiked linearity curve obtained for CTX in liquid milk that was shown to have a good correlation coefficient of 0.9998.

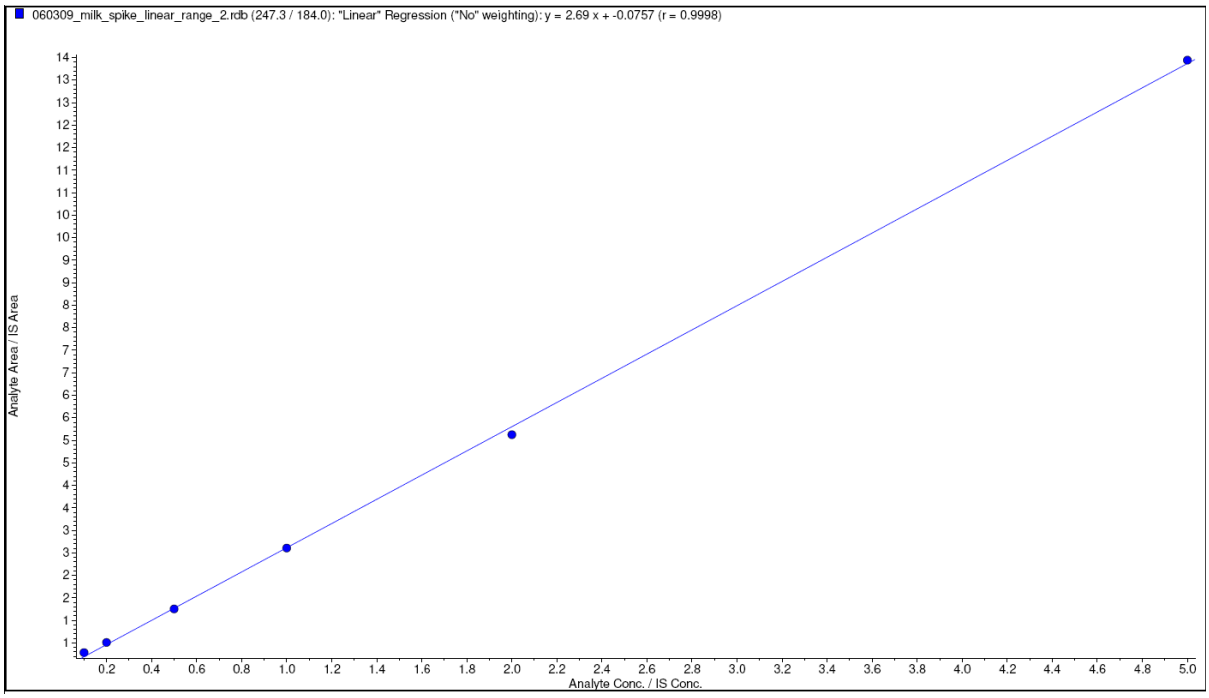

Figure 9.4 Spiked linearity curve for CTX in liquid milk matrix at the concentration levels of 10, 20, 50, 100, 200, and 500 µg/L.

Table 9.7 MDL, MQL values of the various photoinitiators determined in fruit juice and milk matrices (with reference to the respective internal standard) analysed within the range of 10 to 500 µg/L.

Analyte	MDL (ng/ml)		MQL (ng/ml)	
	Fruit Juice	Milk	Fruit Juice	Milk
BP	15.0	15.0	50.0	50.0
ITX	20.0	15.0	60.0	50.0
TX	20.0	20.0	60.0	60.0
DMTX	20.0	20.0	60.0	60.0
CTX	20.0	15.0	60.0	50.0

9.6.4.2 Precision and Accuracy

Precision was assessed at a 50 μg/kg level in both spiked fruit juice and milk samples for 3 consecutive days, with 6 replicates performed each day. The RSD was then calculated by dividing the standard deviation by the mean, and the value multiplied by 100 %. For the spiked fruit juice samples, the RSD for intraday precision (n = 6) ranged from 1.18 – 3.69 %; the RSD for interday precision (n = 3) ranged from 2.11 – 4.66 % (Table 9.8).

Table 9.8 Intra-day (n = 6) and inter-day (n = 3) precision data on fortified spiked juice and milk samples.

Fruit Juice samples						
	Intra-day Precision			Inter-day Precision		
Analyte	**Mean Recovery (%) [n = 6]**	**Standard deviation**	**RSD (%)**	**Mean Recovery (%) [n =3]**	**Standard deviation**	**RSD (%)**
BP	41.15	1.52	3.69	39.58	1.85	4.66
ITX	102.33	1.21	1.18	100.79	2.12	2.11
TX	106.83	1.72	1.61	105.28	2.84	2.70
DMTX	84.12	1.81	2.15	82.01	2.65	3.23
CTX	85.70	1.07	1.25	84.15	2.77	3.29

Milk samples						
	Intra-day Precision			Inter-day Precision		
Analyte	**Mean Recovery (%) [n = 6]**	**Standard deviation**	**RSD (%)**	**Mean Recovery (%) [n =3]**	**Standard deviation**	**RSD (%)**
BP	55.46	1.47	2.65	52.93	2.24	4.23
ITX	91.42	1.02	1.11	89.33	1.81	2.03
TX	105.83	1.47	1.39	102.59	2.94	2.87
DMTX	71.42	1.06	1.49	69.95	2.11	3.01
CTX	94.95	1.46	1.54	92.99	2.09	2.25

The accuracy of the method was assessed at three concentration levels: 50, 500 and 2500 µg/kg in both fruit juice and milk matrices. Six fortified juice and milk samples at each concentration level were extracted and analyzed under the optimized conditions. In both matrices, other than the recovery for benzophenone, extremely good percentage recoveries were obtained (Table 9.9), especially in the fruit juice samples where the method was shown to have slightly improved percentage recoveries in the range of 80.6 – 110.8 %, with excellent

RSD (1.55 – 3.85 %). The recoveries for the milk samples were determined to be in the range of 69.7 – 108.4 % with excellent RSD of 1.61 – 3.01 %. The recoveries for benzophenone tended to be on the lower side consistently for both matrices, ranging from 38.8 - 40.5 % for fruit juice, and 52.5 – 55.3 % for milk. This could be attributed to the use of 1-HCPK as the internal standard for benzophenone, which was of a slightly different structure. The deuterated form of benzophenone was unfortunately not available during this part of the research, which was the reason why it could not be utilized for the MS applications. Therefore, for all subsequent analyses, at least one fortified sample of the same matrix was always analyzed alongside each batch of samples; the final results were corrected for benzophenone, based on the recovery of the analyte in the fortified sample.

As for the other four thioxanthone-related analytes (ITX, TX, DMTX and CTX), ITX-d_7 was utilized as the common internal standard, since the chemical structure of ITX-d_7 was much more closely related to the analyzed substances. Since the recoveries for these analytes were usually within 80 – 120 %, the final analytical results were not corrected for the recoveries.

Table 9.9 Mean percentage recoveries for photoinitiators in both fruit juice and milk samples.

	Mean Recoveries (%) of Fruit Juice samples (n = 6)					
Analyte	**50 µg/kg level**	**% RSD**	**500 µg/kg level**	**% RSD**	**2500 µg/kg level**	**% RSD**
BP	40.48	4.25	39.15	3.83	38.80	3.67
ITX	105.52	3.85	103.50	3.04	105.47	2.37
TX	107.92	2.93	103.83	2.54	110.77	1.81
DMTX	81.37	2.21	80.63	1.55	80.67	1.85
CTX	84.18	3.10	86.47	2.01	80.67	1.61

	Mean Recoveries (%) of Milk samples (n = 6)					
Analyte	**50 µg/kg level**	**% RSD**	**500 µg/kg level**	**% RSD**	**2500 µg/kg level**	**% RSD**
BP	54.29	3.68	55.32	2.41	52.53	2.12
ITX	90.12	2.94	91.93	2.12	89.90	2.37
TX	103.72	3.01	104.50	2.07	108.37	1.81
DMTX	70.32	2.66	70.95	2.44	69.67	1.85
CTX	93.82	2.54	95.43	1.89	92.80	1.61

9.7 Analysis of real beverage samples

The optimized and validated method was tested on a range of 12 fruit juices and milk samples (Table 9.10), which were packaged in heavily printed paper packaging. From the results, benzophenone was the found to be the predominant photoinitiator present in the packaging, followed by ITX, which was detected in 2 of the 7 fruit juice samples. The analysis proved that TX, DMTX and CTX were not detected in the beverage samples, and indicated that they

were not used in the formulations of the ink systems. Nevertheless, the developed method provided the capability to detect these thioxanthone-related analytes at low ppb levels, which would be useful for national food safety programmes.

Table 9.10 Results of photoinitiator content in various beverage samples.

	Content of Analyte (μg/kg)				
Sample	**BP**	**ITX**	**TX**	**DMTX**	**CTX**
Orange Juice A	56.9	< LOD	< LOD	< LOD	< LOD
Orange Juice B	352.9	42.8	< LOD	< LOD	< LOD
Apple Juice	16.7	< LOD	< LOD	< LOD	< LOD
Pink Guava Juice	24.0	< LOD	< LOD	< LOD	< LOD
Carrot Juice	35.0	< LOD	< LOD	< LOD	< LOD
Mango Juice	38.7	< LOD	< LOD	< LOD	< LOD
Mixed Fruit Juice	196.3	37.1	< LOD	< LOD	< LOD
Pure Milk A	44.6	< LOD	< LOD	< LOD	< LOD
Pure Milk B	137.6	< LOD	< LOD	< LOD	< LOD
Chocolate Milk A	70.1	< LOD	< LOD	< LOD	< LOD
Chocolate Milk B	332.0	< LOD	< LOD	< LOD	< LOD
Strawberry Milk	82.0	< LOD	< LOD	< LOD	< LOD

9.8 Conclusions

This method has been successfully optimized for its extraction solvent, the SPE wash solvents and the liquid chromatographic mobile phase gradient in a logical, step-wise manner, and has shown to be suitable for detecting low levels of the five photoinitiators in a number of

different matrixes ranging from acidic fruit juices to fatty milk samples with excellent recoveries at parts per billion levels. Good validation data on precision, accuracy, linearity and robustness have been obtained, which enhances the confidence of using the established protocol on these different matrixes such as fruit juices and milk. The low MDL ranging from 15 to 20 µg/kg, and MQL ranging from 50 to 60 µg/kg established in this simple methodology allow the enforcement of the specific migration limit of 600 µg/L for benzophenone, and 50 µg/kg for ITX, as imposed by the European Union and the Bundesinsitit fuer Risikobewertung (BfR), respectively. This provides utility for both food producers and food safety surveillance institutions.

9.9 References

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Appendix I

Marking	Sample Description	Food Type	No. of cans used	Net Weight / g	Solid portion weight / g	Liquid portion weight / g	Shelf-life during analysis / months
S1	Cooked Ham Jamban cuit	Meat	4	454	1453.7	334.6	unknown
S2	Flakes of Turkey	Meat	8	184	1160.7	279.2	unknown
S3	Baby abalone	Seafood	4	425	825.1	914.5	3
S4	Egg Rolls with Pork	Meat	4	397	1145.7	447.8	3
S5	Skinless sausages	Meat	3	415	781.8	549.4	3
S6	Halal vienna sausages	Meat	3	420	842.6	383.9	4
S7	Cocktail skinless sausages	Meat	3	415	744.0	547.6	3
S8	White Meat Tuna with Spicy Thai Chilli	Seafood	5	185	876.5	24.0	2
S9	White Claims in Brine	Seafood	3	425	537.9	729.4	1
S10	Fancy Pink Salmon	Seafood	4	210	664.2	215.0	8
S11	Sea Asparagus	Seafood	3	425	555.5	726.5	7
S12	Chicken Vegetable Condensed Soup	Meat	4	305	-	-	3
S13	Chicken Corn Chowder	Meat	4	533	-	-	3
S14	Chicken Corn Mutton	Meat	4	340	-	-	3
S15	Chicken Broccoli Cheese	Meat	4	533	-	-	8
S16	Superior Both made in Chicken Ham, Pork	Meat	4	298	-	-	5
S17	Clear Chicken Broth	Meat	4	409	-	-	4
S18	Duck with Preserved Vegetable	Meat	4	370	-	-	3
S19	Corned Beef	Meat	4	340	-	-	2
S20	Oxtail Soup	Meat	4	305	-	-	6
S21	Pork Luncheon Meat	Meat	4	397	-	-	5
S22	Pork Leg with Mushrooms	Meat	4	397	-	-	4
S23	Chaosansi	Meat	8	198	-	-	5
S24	Pork Mince with Bean Paste	Meat	8	180	-	-	9
S25	Stewed Pork Chops	Meat	4	256	-	-	3
S26	Spiced Pork Cubes	Meat	8	142	-	-	4
S27	Stewed Pork	Meat	4	256	-	-	5
S28	White Meat Tuna	Seafood	5	185	-	-	3
S29	Beef Luncheon Meat	Meat	3	320	-	-	5
S30	Corned Pork	Meat	3	340	-	-	2
S31	Chicken Luncheon Meat	Meat	3	340	-	-	3
S32	Mackerel in Tomato Sauce	Seafood	3	425	-	-	2
S33	Sardines in Tomato Sauce	Seafood	6	155	-	-	2
S34	Sardines Chuchee	Seafood	6	190	-	-	1
S35	Fried Sardines in Chilli	Seafood	6	155	-	-	7